

**NON-IONIC SURFACTANT TECHNOLOGY FOR THE DELIVERY AND
ADMINISTRATION OF SUB-UNIT FLU ANTIGENS**

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Thesis Summary

Vaccines have already made a significant impact on global healthcare with the eradication or reduction in disease states; however, further work is required to develop 'safer' vaccines by the use of sub-unit antigens. When considering immunisation, whilst the oral route is the most convenient route for drug and vaccine administration, most vaccines are still delivered via an injection. To be able to be given orally, vaccine antigens need to be associated with carrier systems to both protect the antigen from degradation within the harsh gut environment and to improve uptake by appropriate target sites, namely M cells located in the Peyer's patches, which are responsible for secretory IgA and other mucosal responses. Therefore, niosomes have been considered within this thesis to enhance the protection and delivery of sub-unit vaccines via the oral route and intramuscularly.

Initial work included using Design of Experiments to optimise niosome/ bilosome vesicles prepared from Monopalmitoyl glycerol (MPG), Synthecol (Chol), Dicetyl Phosphate (DCP) and bile salt in terms of their vesicle size, surface charge, suspension pH and antigen loading. Optimisation studies, demonstrated that the ideal composition was a 5:4:1 ratio of MPG:Chol:DCP respectively. Langmuir balance and differential scanning calorimetry studies also demonstrated that, cholesterol inserts between the lipids within the bilayer hence, preventing crystallisation of the hydrophobic tails of the other surfactants.

Using the optimised niosome formulation, oral biodistribution of these niosomes (^3H) and the H3N2 (^{125}I) antigen showed that of the dose administered ($t=1\text{h}$), significantly ($p < 0.05$) higher antigen (59.8%) was recovered within all organs of the gastrointestinal tract (GIT) when formulated with niosome vesicles compared to the free antigen (38 %). Uptake at the target site of the Peyer's patches revealed that on average 1.4 % antigen was present within the Peyer's patches when associated with the niosome vesicles compared to the free antigen dose which demonstrated only 0.4 % antigen was acquired within the Peyer's patches. Furthermore, uptake within the mesenteric lymph tissues demonstrated 0.7 % antigen recovery when associated with niosomes compared to 0.2 % recovery of the free antigen dose. Hence, uptake studies demonstrated that niosome associated antigen show a 2-fold improved uptake and retention at the target sites compared to administering the free antigen alone. Reduction in vesicle size of the niosomal systems to 2 μm made no significant difference in recovery of either the vesicles or associated antigen in the mesenteric lymph tissue. However, when comparing the Peyer's patches, whilst there was no significant difference in localisation of antigen, there was a greater recovery of vesicles within the Peyer's patches of the larger 6 μm vesicles ($p < 0.05$) in comparison to the 2 μm vesicle formulation, which is an indicator of achieving increased mucosal immunity. Within ferrets, the optimised niosomes containing bile salts were shown to lower median temperature differential change and show inflammatory cell counts in nasal washes to be comparable to the commercial vaccine administered intramuscularly after challenge with a clinical H1N1 isolate.

In terms of intramuscular administration, niosomes incorporating H1N1 and H3N2 antigen provided thermostability when stored at elevated temperatures for 3 months at 40 °C confirmed by HAI and ELISA studies when compared to the commercial vaccine. In addition, the association of antigen confirmed the dose sparing ability of niosome preparations to elicit immune responses comparable to the control vaccine doses.

In conclusion, this thesis demonstrates that niosomes can be used as delivery systems for peptides and protein sub-unit antigens by providing antigen protection thereby, enhancing vaccine efficacy when administered orally and intramuscularly.

Keywords: oral vaccine delivery, immunisation, vaccine delivery, niosomes, liposome, lyophilisation.

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List of Publications

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List of Abbreviations

ΔH	change in enthalpy
μ	micron
μg	microgram
μl	microlitre
μm	micrometer
μmol	micromole
Alum	aluminium hydroxide
ANOVA	analysis of variance
APC	antigen presenting cell
BALB	Bagg albino
BCA	bicinchoninic acid
BCG	bacillus calmette-guerin
Chol	cholesterol
Con A	concanavalin A
CPM	counts per minute
CPP	critical packaging parameter
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DCP	dicetyl phosphate
DDA	dimethyldioctadecylammonium
dH ₂ O	distilled water
DRV	dehydration-rehydration vesicles
DSC	differential scanning calorimetry
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DT	diphtheria toxoid
ELISA	enzyme-linked immunosorbent assay
FDA	food and drug administration
G-75	sephadex gel fractionation (3-80 kDa)
GIT	gastrointestinal tract
HIV	human immunodeficiency virus
HPV	human papillomavirus
IM	intramuscular

IV	intravenous
IFN- γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
J/g	joules per gram
kBq	kilobecquerel
LUV	large unilamellar vesicles
MALT	mucosa-associated lymphoid tissues
mBq	megabequerel
MHC	major histocompatibility complex
MLV	multilamellar vesicles
MPG	monopalmitoyl glycerol
mV	milivolts
MWCO	molecular weight cut off
NISV	non-ionic surfactant vesicle
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffer saline
PDI	polydispersity index
PLGA	poly(lactide-co-glycolide)
PLN	popliteal lymph node
SD	standard deviation
SOI	site of injection
SUV	small unilamellar vesicles
TB	tuberculosis
T _c	transition temperature
TDB	trehalose dibehenate
TGA	thermogravimetric analysis
Th	T helper cell
T _m	transition melt
TLR	toll-like receptor
TNF- α	tumour necrosis factor-alpha
UV	ultra violet
WHO	world health organisation

Chapter 1

General Introduction

Publications related to this Chapter:

Wilkhu, J., S. E. McNeil, D. Anderson, Y. Perrie. (2011). "Formulation design considerations for oral vaccines." Therapeutic Delivery **2**(9): 1141-1164. (Wilkhu et al., 2011).

Wilkhu, J., A. Vangala, A. Mohammed, Y. Perrie. (2013). Designing Nonionic Surfactant Vesicles for the Delivery of Antigens for Systemic and Alternative Delivery Routes. Immunomic Discovery of Adjuvants and Candidate Subunit Vaccines. D. R. Flower and Y. Perrie, Springer New York. **5**: 205-232. (Wilkhu et al., 2013).

1. Introduction

1.1 Why vaccines?

Vaccines are essential in healthcare as they can promote protection against infection by a pathogen (Orenstein et al., 2013). For example, vaccines that are available currently include those which offer protection against measles, mumps, tetanus, polio, hepatitis, and Human papillomavirus (HPV) (Ritvo et al., 2005). However, despite continued advances in healthcare where immunisation prevents ~ 2.5 million deaths/year globally, high levels of morbidity and mortality are still caused by infectious diseases (Orenstein et al., 2013, Liu et al., 2012). This is due to the underlying problem that for many infectious diseases, there are low efficacy or no vaccines available (e.g. Human immunodeficiency virus (HIV), Tuberculosis and malaria). However a second consideration in the development of vaccines is accessibility; whilst well developed public health strategies are in place in many developed countries, many areas of the world still lack the required infrastructure to support wide-scale public health vaccine programmes (Di Fabio and de Quadros, 2001, Graham et al., 2012). To support this, accessibility to vaccines is a key requirement; cost, the ability to transport vaccines without the need for cold-chain transport, and the ability to administer the vaccine without the needs of needles would make a major impact on improving accessibility (Mitragotri, 2005, Giudice and Campbell, 2006, Levine, 2003). Therefore, it is essential to maintain research in vaccines to develop new and improved vaccines including novel methods of vaccine delivery which could address the above outlined challenges.

1.2 Vaccine classification and their limitations

An effective vaccine can be described as one which prevents infection and disease with the capability of eliciting specific immune responses with the aim of providing

protective immunity (Bramwell and Perrie, 2005). In general, vaccines should satisfy certain criteria in order for it to be classed as a good vaccine and these criteria include:

1. the capability of inducing the correct immune response,
2. the safety of administration (stable),
3. increased patient compliance by offering alternative routes of delivery and/or the potential of needle-free administration.

To achieve the above outcomes, a range of vaccines have been developed and these may be divided into three categories; live attenuated, inactivated and sub-unit vaccines depending upon their properties.

1.2.1 Live vaccines

Live vaccines (also referred to as attenuated vaccines) promote strong immune protection; they are very effective in initiating mucosal and humoral immune responses as they are able to mimic a natural infection (Webster et al., 2003). The ability of live attenuated vaccines to replicate intracellularly allows sufficient quantities of the antigenic peptides to be produced which are represented by MHC-1 molecules, in turn activating cytotoxic T lymphocyte (CTL) responses (Webster et al., 2003, Burgdorf et al., 2007). However, in general live vaccines tend to have higher associated risks compared to other types of vaccines; there is an associated risk for live attenuated vaccines to revert back to the wild form, posing as a risk for inducing a disease state especially to immunocompromised individuals (Chadwick et al., 2010). However, whilst in general live vaccines have proven to be very effective this has not to be the case for all. For example, *Mycobacterium tuberculosis* (TB) is an infection that affects the population across the world with about 1.7 million people dying each year from the infection (Ryan et al., 2011). Currently, there is a vaccine available for tuberculosis based on the attenuated strain of the *Mycobacterium bovis* (BCG) however, only offers

protection against some forms of the strain (Holten-Andersen et al., 2004). Using attenuated strains have the advantages of producing a full immune response and is generally cost effective as there is a long duration of immunity. The problem above is that by using the live attenuated strains of viruses is that mutations can lead to a reversion to virulence.

1.2.2 Inactivated vaccines

As an alternative to attenuated vaccines, inactivated vaccines have been developed where microorganisms have been inactivated by the use of heat or chemicals such as formaldehydes (Perrie, 2006, Baxter, 2007). The use of inactivated vaccines offer advantages in terms of reduced side effect profiles compared to live vaccines; however, inactivated vaccines tend to have reduced efficacy as they are unable to multiply in the host to give a strong signal to the adaptive immune system hence, further doses are required when using inactivated vaccines (Baxter, 2007).

1.2.3 Sub-unit vaccines

A third group of vaccines are sub-unit vaccines. These are based on synthetic protein or peptide-based antigens and therefore offer major advantages in terms of improved safety profiles compared to other types of vaccines. However, subunit vaccines are limited by their poor immunogenicity and are unable to elicit strong CTL responses when administered without adjuvants or compared to live vaccines (Vangala et al., 2006, Perrie et al., 2008).

As mentioned, subunit antigens are weak immunogens and therefore generally require the use of adjuvants. Adjuvants augment the effects of vaccines due to the increased stimulation of the immune system in its response to the vaccine, allowing increased immunity to a specific disease (Schijns, 2000). The inclusion of an adjuvant within the

vaccine formulation can enhance immunological memory and coverage, and allows for antigen sparing and reduced number of doses (Tritto et al., 2009). Currently there are only a handful of adjuvants licensed for human clinical use. One of the oldest adjuvants is Alum which, since the 1920s, has been incorporated into various human vaccines as an aluminium salt such as aluminium hydroxide ($\text{Al}(\text{OH})_3$) and aluminium phosphate (AlPO_4). For human use, USA only approved the aluminium salt adjuvants for over 80 years, however, more recently ASO₄ (oil-in-water emulsion combining Alum and Monophosphoryl Lipid A) has been approved by European regulators and by the FDA as an adjuvant for vaccines (Baldwin et al., 2012, Mbow et al., 2010). In addition, European regulators have approved and licensed MF59 (squalene oil in water emulsion) as an adjuvant in e.g. a flu vaccine known as Flud. Despite these systems, further adjuvants are needed. Pre-clinical studies and clinical studies have shown that alum is often less potent than adjuvants such as the oil in water emulsion ones and has shown to be a poor inducer of protective Th-1 immune responses which are crucial for protection against intracellular pathogens (Mbow et al., 2010). Adjuvants will be discussed in further detail within section 1.8.

1.3 Oral vaccines

Currently, most of the vaccines available are injectables which have several disadvantages including irritation at site of injection which causes a lack of patient compliance. Moreover, needle phobia is a concern in adults and children as immunisation can then become a stressful procedure (Breau et al., 2001). When introducing herd immunity, patient compliance is essential as a large percentage (generally >95 %) of the population needs to be immunised. Vaccines delivered via parenteral injection need to be stored in a fridge during transport, therefore needle-based vaccines cause issues in relation to cost, efficiency and logistics. Furthermore,

trained personnel are required when administering the vaccines which may not be possible in certain countries. In addition, the injuries associated with needles and the reuse of needles and syringes in developing countries is leading to unsafe injection practices. For example, the number of HIV infections resulting from the reuse of needles by health care providers was reported by Kane et al, (1999) of being 80,000-160,000 (Kane et al., 1999). Therefore it is essential to find a new delivery route for vaccines with needle free immunisations being an important goal.

The oral route is the preferred route of delivery due to its non-invasive structure and low infection risk and, most importantly, the mucosal immunity can be promoted by oral vaccines by offering strong resistance against many pathogens that infect via the mucosal lining (Clark et al., 2001). Furthermore, the safety profile of oral vaccines is more acceptable as side effects such as flu like symptoms, fever and diarrhoea associated with parenteral formulations are reduced or absent (Santiago, 1995). The efficacy in the elderly could also be improved as the mucosal associated lymphoid tissue (MALT) does not diminish through the ageing process (Santiago, 1995). However, there is conflicting data where researchers suggest that the mucosal immune response in the GIT is compromised by ageing (Schmucker et al., 1996). A review by Ogra (2010) suggests that ageing reduces the number of Peyer's patches which are required for the uptake of antigens and carrier systems (Ogra, 2010). The M cells located in the Peyer's patches are responsible for secretory IgA and other mucosal responses, hence if there is a decrease in numbers of Peyer's patches due to ageing, the production of secretory IgA will also decrease (Schmucker et al., 1996). There are animal models to understand the ageing immunity, however, due to the lack of elderly human data on ageing mucosal immunity the implications of ageing remain to be defined.

In general, even though the oral route is the most accessible and has good patient compliance, only a small number of vaccines can be administered via the oral route due to problems with vaccine stability within the gastro-intestinal tract (GIT). This results from metabolism and degradation of the vaccine occurring pre-systemically in the GIT, where low pH and protease enzymes promote degradation (Russell-Jones, 2000). In addition, the residence time of the vaccines at the immune induction sites within the GIT have short exposure times due to normal GI transit. Due to this short exposure time, higher doses or an increased frequency of dosing would be required to supply sufficient antigen to elicit an immune response (Webster et al., 2003). However, it is also essential to avoid promoting systemic tolerance and low secretion of antigen specific IgA through excessive dosing with the vaccine (Mowat, 2003, Russell-Jones, 2000).

In relation to the above points, an oral polio vaccine was developed in 1958 by Dr. Albert Sabin where he attenuated the wild polio virus. The most common form of oral polio vaccine (OPV) is the trivalent vaccine, which means that it contains live attenuated strains of the three serotypes of poliovirus (Fine and Carneiro, 1999). As attenuated vaccines show transient growth an advantage of OPV is that it allows prolonged exposure of the immune system to the attenuated organism. This results in increased immunogenicity and allows the cells to enhance their memory, hence making the OPV superior to the traditional inactivated injectable polio vaccine (IPV). Both vaccines produce antibodies in the blood, with the OPV also producing a local immune response with the release of SIgA within the mucosa. The extra mucosal immunity achieved by the body indirectly protects other individuals in the community who have not been vaccinated, which is advantageous as it inhibits the spread of the polio virus. As a result, the OPV confers long lasting immunity which is one of the important

criteria in making a vaccine successful. However, the main limitation of OPV is the risk of the attenuated form of the polio virus reverting back to the virulent form; its replication in the GIT can cause it to mutate into various neurovirulent forms, hence back to its wild-type sequence which can induce polio in humans (Troy et al., 2011). This is a major problem in developing countries, as other GIT viruses can interfere with the replication of the attenuated polio vaccine virus in the intestine of the vaccines (Fine and Carneiro, 1999).

Indeed, the use of OPV can cause vaccine-associated paralytic poliomyelitis (VAPP), either in the vaccinees or people who are in close proximity to them (Troy et al., 2011). The OPV mutations are associated with VAPP and more recently, prolonged replication of the OPV can cause up to 15 % mutation to a vaccine-derived poliovirus (VDPV). This in turn, can cause outbreaks of poliomyelitis (Minor, 2009). VDPV revertant strains are reported to be transmissible and hence pose significant population risks (Hull and Minor, 2005). Thus in industrialised countries, IPV is now more widely used to prevent risk of outbreaks from regular use of OPV and its mutant forms (Troy et al., 2011, Heinsbroek and Ruitenbergh, 2010).

Therefore, due to specific problems associated with the oral route and the use of live vaccines such as OPV, researchers have used various strategies to overcome these barriers which will result in potential effective oral vaccines. These strategies include developing particulate carrier systems which use sub-unit vaccines instead of live or attenuated vaccines and developing carriers which protect the macromolecule from the enzymes in the GIT from degradation and metabolism and other epithelial barriers which are discussed in Section 1.5.

1.4 Mucosal vaccination

The mucosal surface area is 400 m² (Baudner and O'Hagan, 2010) and the main functions of the mucosal immune system are to:

1. Protect the mucous membrane from invasion and colonisation of potentially harmful microbes,
2. Prevent the uptake of foreign proteins derived from food sources or airborne matter,
3. to prevent the development of a harmful immune response to (2) if they reach the site (Holmgren and Czerkinsky, 2005).

The MALT acts as a physical barrier between the external environment and the internal cavity of the body, protecting and preventing invasion by bacteria, foreign pathogens and micro-organisms. However, due to the large surface area of the overall mucosal surface, it is highly susceptible to many pathogenic microorganisms, and therefore has a highly specialised immune system in order confer immune protection. The immune system is adapted to establish a difference between antigens, pathogens and vaccines that enter via mucosal surfaces to those entering via the blood supply or through tissues (Neutra and Kozlowski, 2006). It is essential to develop vaccines which target the effective cells required to achieve immunity. For example, injected vaccines are poor inducers of mucosal immunity hence, are less effective against infection at mucosal surfaces (Levine, 2000). Mucosal immune responses are initiated via the MALT which is comprised of several sites which include Peyer's patches, Mesenteric Lymph Nodes (MLN), appendix, tonsils and adenoids in the respiratory tract (Kiyono and Fukuyama, 2004). Within this thesis, one of the aspects will concentrate on vaccine delivery systems to the Peyer's patches which contain specialised epithelial cells known as the Microfold 'M' cells which capture the antigens and subsequently process the antigen to local APC's such as dendritic cells, macrophages and B lymphocytes (Figure 1.1)

(Bilsborough and Viney, 2004). M cells are specialised epithelial cells that deliver antigens from the lumen of the gut to the epithelial lymphocytes and subsequently to the subepithelial lymphoid tissues (Mitragotri, 2005).

Orally delivered vaccines are recognised by the M cells within the Peyer's patches which uptake the antigen and deliver it to the dendritic cells which reside at this location (Holmgren and Czerkinsky, 2005). The immunological response of the dendritic cells has been reviewed in detail by Coombes and Powrie (2008), where findings indicate that dendritic cells within the Peyer's patches produces higher levels of Interleukin-10 (IL-10) than those dendritic cells present within the spleen (Coombes and Powrie, 2008). In addition, the CD4⁺ T cells which are activated by the Peyer's patches produce higher levels of IL-4 as well as IL-10 (which are represented by a T helper 2 phenotype) than those of the spleen dendritic cells (Iwasaki and Kelsall, 1999).

Figure 1.1 shows that particulates and subunit antigens are taken up by the M cells or epithelial cells which predominantly consists of enterocytes and mucus secreting goblet cells. M cells have a sparse, irregular microvilli on their apical surface in addition to the presence of a basolateral cytoplasmic invagination which creates a pocket containing lymphocytes and macrophages (Clark et al., 2001). These features of the M cells are believed to establish mucosal immunity by allowing a route for antigens to be delivered. This route delivers the antigens to underlying lymphoid tissues where a secretory immune response is initiated. The epithelial cells play a major role in mucosal defence as they are able to detect foreign bodies through pattern recognition receptors such as Toll-like receptors (TLRs). The release of cytokine and chemokine signals to the dendritic cells and macrophages which underline the mucosal cells as shown in Figure 1.1, allow an innate and adaptive immune response to be triggered (Kagnoff and

Eckmann, 1997, Izadpanah et al., 2001, Kraehenbuhl and Neutra, 2000, Neutra and Kozlowski, 2006).

Although the mechanism of action upon absorption by the Peyer's patches remains unknown, the M cells remain good targets for subunit particulate and other oral vaccine delivery systems. However, tolerance and mechanisms of uptake still need to be resolved and determined. This leads on to the next topic of barriers and strategies of delivering vaccine delivery systems to the Peyer's patches and M cells.

Induction of a mucosal response

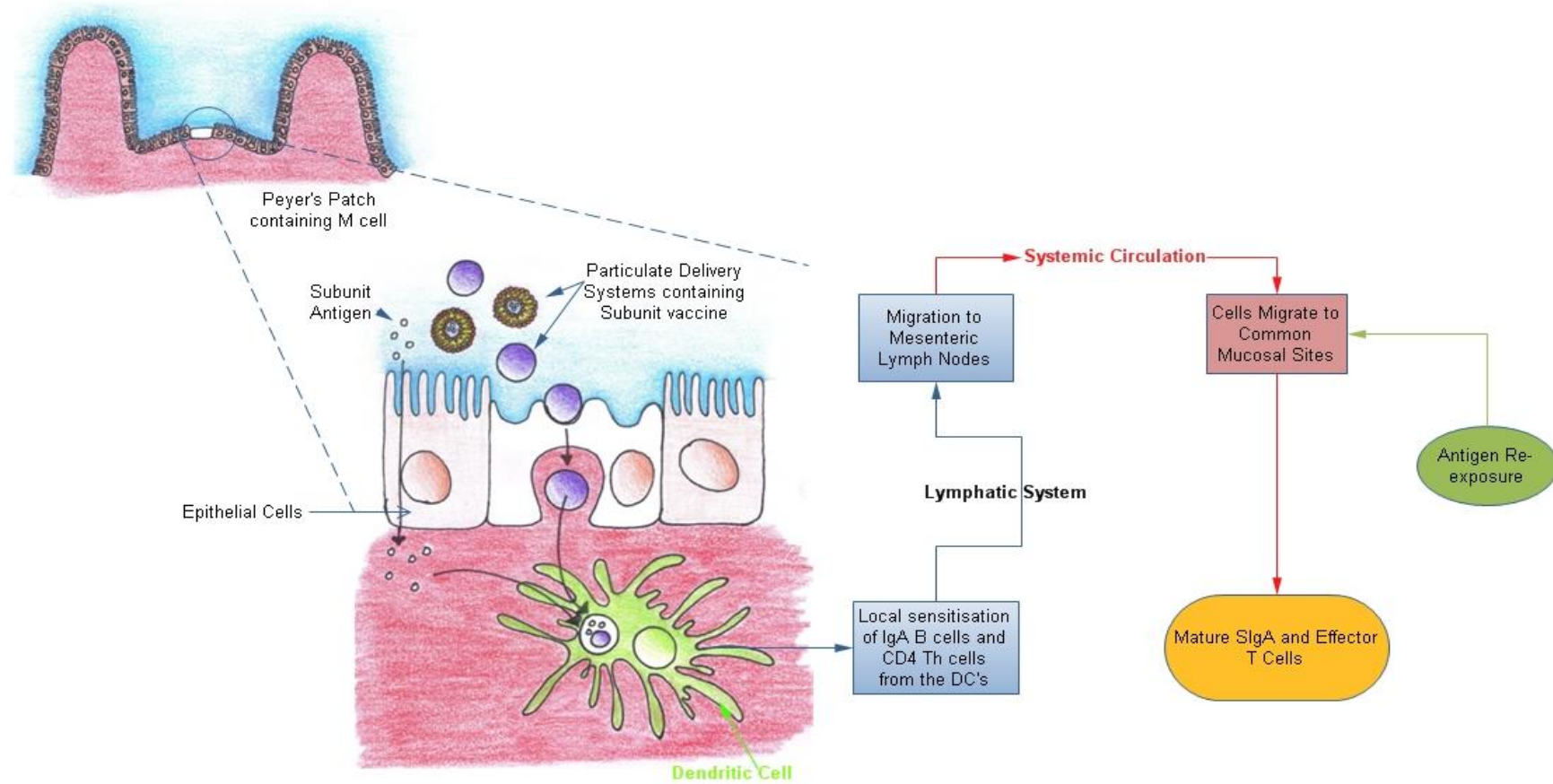


Figure 1.1: Sequence of events which take place after the antigen is ingested and reaches the mucosal surfaces within the small intestine. (Kiyono and Fukuyama, 2004, Mitragotri, 2005)

1.4.1 Barriers and strategies for effective delivery to M cells

The small intestine is the site of absorption for electrolytes, nutrients and fluids. However, it also acts as a barrier which prevents the absorption of potentially toxic or harmful substances. In order for vaccine particulates to be absorbed in the small intestine, they must penetrate through two barriers which include the mucus gel layer and the mucosa. The intestinal mucosa comprises epithelial cells and their associated glands and mucus layer which is a secretion of the high molecular weight glycoprotein and other non-specific defences including mucins and antimicrobial proteins (Kraehenbuhl and Neutra, 2000). These structures of the mucosal lining trap enzymes and create an environment which is highly degradative to substances it comes in contact with (Clark et al., 2001). This layer has a major role in protecting the mucosa from harmful bacteria, pathogens or chemicals. In addition to protecting the mucosa the mucus layer maintains the pH difference that is observed between the GI lumen and the mucosa. Therefore, the mucus layer will hinder the diffusion of certain compounds and as a result size, pH and electric charge will be major parameters when targeting delivery of oral vaccines to these areas. As well as the mucus layer acting as a diffusion barrier, it also acts as a physical barrier by preventing the absorption of particles. The mucus entraps the particles, causing agglomeration in the mucus layer which then causes an increase in net size and a decrease in the diffusion coefficient through the mucus layer. The surface charge has a significant impact upon the uptake of particulate delivery systems e.g. studies by Tabata and Ikada (1988) have shown that the peak uptake of particles occurs with a zeta potential of -70 mV (Tabata and Ikada, 1988). This zeta potential also shows excellent stability and results in no coagulation of the particles due to the high surface charge (Freitas and Müller, 1998, González-Rodríguez and Rabasco,

2011). As a result zeta potential studies should be aimed to achieving such values to enable them to be taken up by the membranous cells.

1.5 Approaches for delivery systems (particulate delivery systems)

Fundamentally for the delivery of soluble antigens, carrier systems should be designed with the target site in mind, therefore for oral vaccines these carriers must be sufficiently acid/enzyme resistant, and formulated to promote uptake by the GALT. To allow efficiency to be achieved, in terms of eliciting an immune response and providing mucosal immunity by mucosal vaccines, particulate delivery systems can be employed as presented in figure 1.2. Ideal properties of particulate delivery systems should be able to successfully target the mucosal sites (in this case, Peyer's patches) and to allow specific interactions to take place at these sites to initiate an immune response. When considering particulate uptake, there are a range of factors which have been identified as key parameters including: size, charge and hydrophobicity of the particulate, designing the systems to be muco/bioadhesive, and the addition of targeting moieties and adjuvants to the system.

Although there are several mucosal routes of administration, such as oral, intranasal, inhalation and intravaginal of antigens (Holmgren and Czerkinsky, 2005, Mitragotri, 2005) this thesis will be focussing on oral delivery of vaccines to the specialised epithelial cells of the small intestine and intramuscular delivery of influenza vaccines. Mucosal tract epithelial cells vary from site to site within the body with the composition of the APCs and lymphocytes also vary at these sites hence Peyer's patch epithelial cells will be the main focus of targeting (Baudner and O'Hagan, 2010).

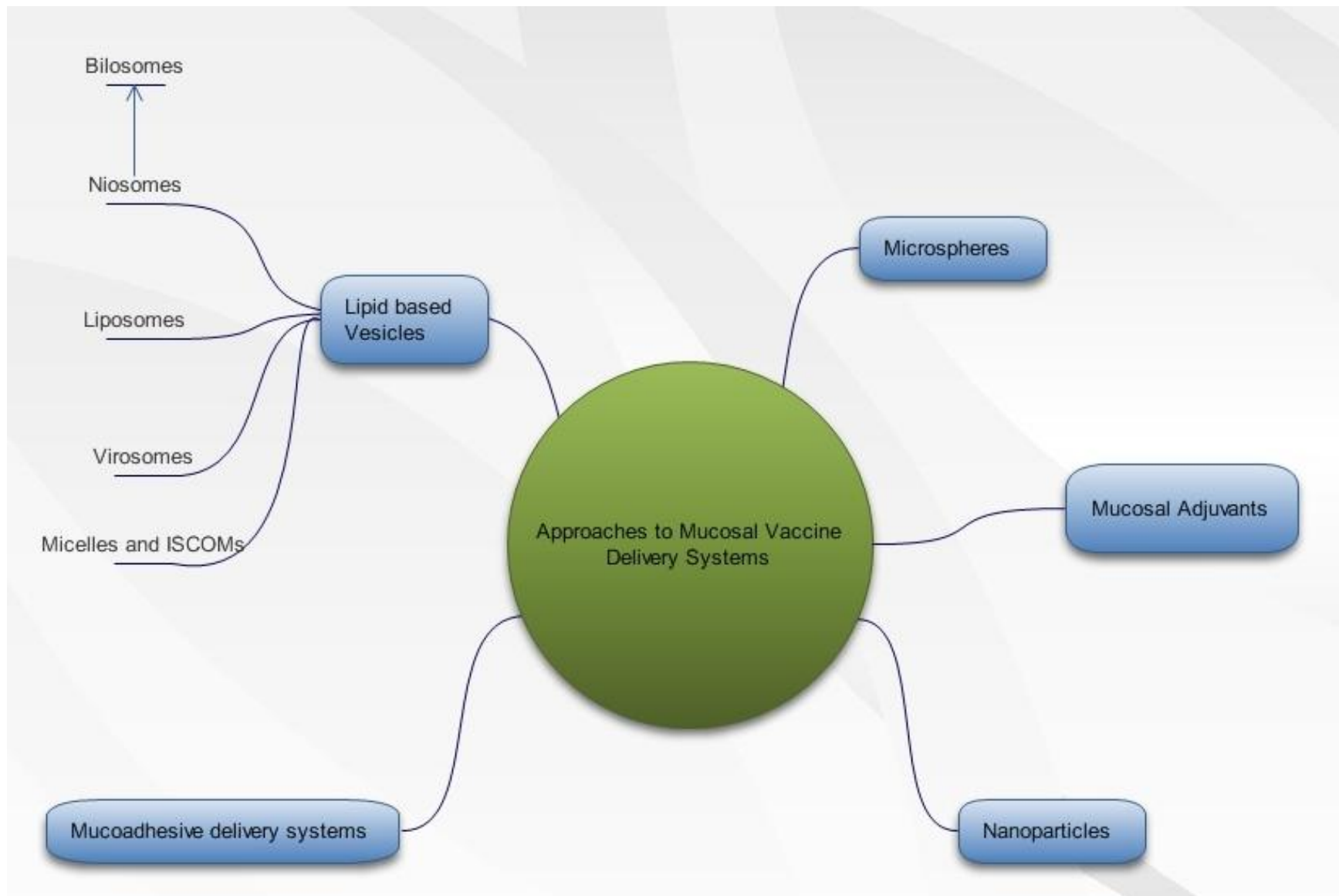


Figure 1.2: The current particulate approaches to mucosal delivery systems

1.5.1 Microspheres

Microspheres are small particles with large surface area to volume ratios, which have a good potential to deliver drugs. Generally, microspheres are solid, approximately spherical particles ranging in size from 1-1000 μm (Lu et al., 2007). Microspheres for vaccine delivery have been useful in the delivery of antigens which are ingested by the immuno-competent cells, in turn providing controlled antigen release and lasting immunity. Microspheres have been reported to protect the antigen from intestinal bile salts, the variation in pH along the GIT and from the degradative enzymes (Hanes et al., 1995, O'Hagan and Illum, 1990).

Several studies with microspheres have shown that antigens encapsulated in microspheres can elicit secretory IgA and circulating antibody IgG in comparison to unencapsulated antigen (Langer et al., 1997). Studies by Eldridge et al, (1989); Shakweh et al, (2005) and a review by O'Hagan (1990) have suggested that the Peyer's patches readily uptake particles less than 10 μm in size and prefer hydrophobic surfaces. Particles within the size range 3 - 10 μm reside within the lymphoid tissue of the Peyer's patches (Florence, 2005). In addition to vesicle size, surface charge and hydrophobicity are also crucial factors in the uptake of microparticles via the Peyer's patches, with both being essential as particles have to translocate between a hydrophobic mucus layer and then across a hydrophilic interior of the cell (Hussain et al., 2001). In terms of hydrophobicity, Norris and Sinko in 1998 established its role through the intestinal mucin and Caco-2 cell monolayers; different poloxamers were coated onto microspheres to promote different hydrophobicities and reduce the contact angles between the particle/water phase (Norris et al., 1998). Using this range of microspheres the authors were able to demonstrate that as the hydrophobicity of the microspheres increased better diffusion is observed through the mucus layer. However,

this enhanced diffusion effect then decreases through the hydrophilic environment of the cellular cytoplasm (Hussain et al., 2001). However, despite the range of advantages microspheres offers, one of the major problems hindering the progression of microspheres as carriers for vaccine formulations for human use is stability of the antigen during microencapsulation, storage and release (Cleland, 1998).

1.5.2 Nanoparticles

Nanoparticles are colloidal carriers which range in size between ~10-1000 nm and is a fast developing field offering several advantages for potential oral vaccine delivery. Whilst generally this could include liposomes, micelles, etc most specifically it can refer to polymeric solid particulates within the nano range. With such nanoparticles the antigen or vaccine components can be adsorbed onto the surface of the nanoparticles or dispersed within the matrix (Vauthier and Bouchemal, 2009). In addition to nanoparticles, nanocapsules can be used where the antigen is fully encapsulated within the nanocapsule within the core. The core can either be aqueous or oil based depending upon the antigen to be used, hence allowing lipophilic or hydrophilic antigens to be incorporated (Vauthier and Bouchemal, 2009). Oral administration of antigen-loaded nanoparticles can promote antigen stability within the particulate as it transits through the GIT due to protection. Alongside stability and protection, the polymers used to formulate nanoparticles can be modulated to control physicochemical characteristics such as zeta potential and hydrophobicity. In a review by des Rieux et al, (2006) it is suggested that in addition to the above advantages, the drug/antigen release properties and biological behaviour of the nanoparticles can also be modified (des Rieux et al., 2006). The resulting nanoparticles are subsequently taken up by the epithelial cells within the mucosa, M cells and Peyer's patches (represented in figure 1.1) (Gelperina et al., 2005).

The materials used to form the particles must be non-reactive and compatible with a broad range of excipients as well as their availability in pharmaceutical grade purity (Bhavsar and Amiji, 2007, Bhavsar and Amiji, 2007b) and various materials can be applied in the formulation of nanoparticles including polymers, lipids, proteins and metals (Chadwick et al., 2010). When considering the construction of such nanoparticles, the main consideration for oral delivery involves finding equilibrium between the desired surface properties and the matrix used in the formulation. These factors control nanoparticle uptake and stability; hence it is vital to establish a relationship between these two factors. Nanoparticles work on a mechanism of releasing their loaded antigen either by particle degradation, erosion, diffusion out of the matrix or swelling (Gelperina et al., 2005). Due to these release mechanisms, the particulates must be sensitive to the local milieu of the environment such as pH, temperature and enzymes including the presence of other particulates such as food.

The size of the nanoparticles also plays an important role as colloidal instability leads to aggregation and flocculation. Similarly the chemical stability of a nanoparticle matrix is crucial for its biodegradability and release of the encapsulated antigen (Florence, 2005). A recent review by Chadwick et al (2010) studies, in depth, the different polymers available for producing nanoparticles, and how the size of the particulates vary depending on the polymer used and its properties. The review also suggests how polymers can be used to create micelles and complexes of micelles to potentially deliver antigens orally with a possibility of improved bioavailability. Micelles in general, due to the lack of covalent bonding, have the potential to dissociate especially under acidic conditions. This is good when delivering a drug to an acidic compartment, such as the stomach, but in terms of oral vaccine delivery to the mucosa, this will not be useful as there will be premature release of the antigen. However in general, due to the

lack of comparative studies available, there is no conclusion regarding which polymers or other formulation factors are the most efficient for nanoparticles in oral delivery. Therefore, due to the multitude of parameters which influence the uptake and facilitation of nanoparticles in oral delivery there is no general establishment for the non-specific uptake into M cells or enterocytes.

1.5.3 Bioadhesive delivery systems

As previously mentioned, the primary function of mucosal vaccination relies on the movement and transport of the antigen along the mucosa, through the barriers and finally to be taken up by the APCs. Bioadhesion is a technique employed to prolong residence time of particulate delivery systems by targeting the specialised mucosal M cells and adhering to the surfaces. This mechanism of action can allow better uptake of the antigen by the APCs, therefore providing an effective means of mucosal vaccine delivery (Brayden et al., 2005, Kuolee and Chen, 2008). Bioadhesive delivery systems would still require the use of immunological adjuvants to provide an increased immune response. Mucoadhesion is another term used to refer to bioadhesion. However, generally this describes the interaction of delivery systems and the mucosal surfaces. In terms of vaccine delivery, studies carried out by O'Hagan et al, (1993) suggest that mucoadhesives contribute to an immune response by several means which include increased duration of retention at mucosal site, greater interactions with the mucosal epithelium sites, enhanced absorption and the possibility of achieving sustained release from the particulate systems (O'Hagan et al., 1993, Baudner and O'Hagan, 2010).

Currently there are several bioadhesives used within the vaccine field which include starch (Rydell et al., 2005), chitosan (Arca et al., 2009, Amidi et al., 2010), hyaluronic acid (Singh et al., 2001), carbopol (Kockisch et al., 2003), alginates and lectins (Lavelle

et al., 2002, Lavelle et al., 2004, Jepson et al., 2004). Factors which influence these bioadhesives and the polymers include the molecular weight, cross-linking and packing, concentration, flexibility and the degree of swelling the polymer undergoes upon hydration. In addition to these factors, the antigens used within these bioadhesive particulates must be compatible in terms of charge interactions and the solubility of the antigen in relation to the solubility of the polymer. As a result, bioadhesive systems become a very complex system with various parameters that need to be addressed in addition to the particulate size, charge and hydrophobicity.

In various research studies into these bioadhesive systems, bioadhesive particulate delivery systems have shown to be successful in animals such as mice; however, when up-scaled to human trials these systems become ineffective. For example a study by Rydell and Sjöholm (2005), where microparticles made from starch, show promising results as an adjuvant carrier in mice (Rydell and Sjöholm, 2005). The results of the experiment show that mucosal immunity and systemic immunity was achieved when oral dosing starch microspheres in mice. Further to this, Stertman et al (2004) challenged the mice with live bacteria and successful protection was achieved (Stertman et al., 2004). However at a later stage when Rydell et al (2006) prepared the vaccines for human trials, the vaccine was not effective (Rydell et al., 2006). There could be a number of possible reasons for the ineffectiveness such as differences in the GIT between humans and mice, the longer transit time in humans in relation to mice and the differences in the barriers such as thickness of mucous, interference of food particulates and other enzymes present. Furthermore, alginate microparticles have shown to induce protective immunity against specific bacteria where intestinal IgA antibody responses are achieved by alginates delivered orally (Kim et al., 2002, Sun et al., 2007).

Several recent reviews have been carried out on bioadhesives with an overall review carried out by Baudner and O'Hagan (2010) and further in depth reviews on specific bioadhesives such as the chitosans by Arca et al (2009) and Amidi et al (2010). In terms of bioadhesive particulates as a carrier for antigens, there are promising microparticulate formulations available in a broad range of animals using different antigens for the oral route. However, there is such a diverse range of properties effecting the transit and mechanisms of antigen release from these systems that no conclusion can be formed in terms of what is the best design of a bioadhesive particulate delivery system. In terms of having a complete delivery system based upon bioadhesive particulates, the increased residence time should not be deemed the most important parameter to focus on as vesicle size and charge also play a major role in protective immunity via the oral mucosal route.

1.6 Lipid based systems

As previously mentioned, there are a range of particulate constructs that are being considered for drug and vaccine delivery including solid particulate systems (which can be built from polymers, lipids, proteins, etc) and bilayer type vesicles (which are built from molecules with surfactant type properties). Of these, liposomes are the most well known and investigated after their early recognition as vesicles by Bangham (Bangham and Horne, 1964) and their first use as delivery systems by Gregoriadis et al (1971). However, a wide number of variations, based on these initial liposome systems, have been developed including stealth liposomes to improve circulation profiles (Lasic et al., 1991), vesicles built from non-ionic surfactants also known as non-ionic surfactant vesicles (NISVs) or niosomes (e.g. for cosmetics (Handjani-Vila et al., 1979) or as drug/antigen carriers (Azmin et al., 1985, Baillie et al., 1985), surfactant polymers (e.g. polymersomes Okada et al., 1995), cationic systems which can electrostatically bind

DNA (e.g. lipoplexes Felgner et al., 1987), vesicles incorporating bile salts to improve stability (e.g. bilosomes Conacher et al., 2001), or virus components (e.g. virosomes Almeida et al., 1975) to name but a few. Many of these systems use alternatives to phospholipids to circumvent potential issues related to storage instabilities and cost (e.g. synthetic based systems), to improve stability within harsh biological environments (e.g. bilosomes and polymersomes), or alternatively to modulate the properties of the vesicles in terms of immunological efficacy (e.g. virosomes).

1.6.1 Liposomes as vaccine adjuvants

Similar to polymers, lipids are already used in a wide range of commercial product and there are a variety of carrier systems that can be formulated from lipids, with liposomes probably being the most commonly thought of, given the substantial body of research investigating them. However, there are various types of lipid employed in formulations including non-polar lipids and polar lipids, with the later being sub-divided into 3 classes and of these class II being used to formulate liposomes and class III lipids for micelles. Liposomes (bilayer vesicles generally composed of phospholipids ranging from around 50 nm to several microns in size) were identified as being effective immunological adjuvants by Allison and Gregoriadis (1974) where the ability of negatively charged liposomes (prepared with the inclusion of dicetyl phosphate) to deliver and potentiate immune responses against diphtheria toxoid (DT) was demonstrated; from this work a plethora of investigations have subsequently steamed. The main cited advantage of bilayer vesicles over microspheres is the ability to easily formulate liposomes in a range of sizes with the choice of lipids dictating the liposomes surface properties. Like polymer systems, there is a wide range of options for manufacturing liposomes with lipid hydration (based on the original work of Bangham, 1968) remaining the simplest and most commonly employed method. After production

of multilamellar vesicles (MLV), the vesicles can easily be manipulated to control their size and also their antigen loading depending on methods adopted.

Liposomes have been used in vaccine technology as carriers of antigens, which are either encapsulated into the aqueous space, incorporated into the bilayer of the liposomes or associated with the surface of the vesicles. Liposomes as carriers of antigens allow a reduction in biodistribution but enhance targeting which are vital for immunotherapy (Gregoriadis et al., 1999). There are many advantages through the use of liposomes for vaccine delivery which include:

- Protection of drug or antigen from the external milieu of the body such as the GIT, due to the encapsulation process. Also, encapsulation of an antigen into liposomes can reduce the toxic effects that the antigen exerts in the body upon administration.
- Liposomes themselves are generally non-immunogenic therefore allowing suitable systemic and non systemic administrations (Perrie et al, 2008).
- Variation in liposome size, allows for a broader range of administration routes, e.g. nasal, oral, intramuscular, subcutaneous or intravenous.
- Formulation of liposomes can take place in many forms such as suspensions, aerosols, gels, creams and lotions. This is crucial as immunisation via the parenteral injection is less economical for a mass immunisation; trained personnel and sterile needles are required which poses cost issues. Needle-free vaccinations in turn, will also have better patient compliance.

Cationic liposomes have been studied by Perrie et al (2002) where DNA is either entrapped within the bilayers or by a complexation process onto the surface of the liposomes. These cationic liposomes result in a positively charged carrier hence

allowing electrostatic interactions between the vesicle and the negatively charged cell membrane, resulting in intracellular delivery of the DNA (Kirby et al., 2008, Rosada et al., 2008). Studies carried out by Szentkuti et al (1997) showed that nanosized cationic dyes become entrapped within the negatively charged mucus, hence this could aid retention at the cell surface (Szentkuti, 1997).

Lipids have also been the foundation for use as polymerised liposomes or ISCOMS where the use of polymerised liposomes has been developed and tested which stabilise the vesicles to be used orally (Chen et al., 1996, Gaucher et al., 2010). The generation of polymerised liposomes involves creating cross linked networks between the liposome membranes which provides stability through the GIT. Studies carried out by Chen et al (1996) have shown promising data in protecting the antigen from GIT enzymes and degradation. These systems then behave similarly to micro and nanoparticulates where polymers are used to protect antigens; however, the release mechanisms then vary depending upon the type of polymer used, and its degree of crosslinking and other physiochemical properties. Lipid based delivery systems can also form immune stimulating complexes (ISCOMs) where they form 3 dimensional cage-like structures. ISCOMs are generally in the size range similar to viruses of 30 – 70 nm in diameter and are composed of phospholipids, cholesterol, antigen and the saponin adjuvant Quil A (Copland et al., 2005, Maloy et al., 1994). Saponins comprise powerful immunostimulatory activity and then by combining the advantages of particulate delivery systems alongside incorporating the antigen forms the basis of ISCOMs and as a result can be used to delivery antigens orally. Studies by Mowat and Maloy (1994) have shown that hydrophobic antigens readily incorporate into ISCOMs, whereas hydrophilic antigens require modification before they can insert into the system. Using

ISCOMs to deliver antigens promotes higher induction of Th1 and specific CTL responses in comparison to micelle formulations (Sjolander et al., 2001). Clinical trials for use in human vaccines are currently ongoing to test the safety, efficacy and toxicity profile (Copland et al., 2005).

For oral vaccine delivery, lipid based vesicles such as liposomes can be used as effective carriers of macromolecules, however, when in the GIT they are exposed to intestinal bile salts. The intestinal bile salts cause the membrane of the lipid vesicles to deform and lyse resulting in the release of macromolecules from the vesicle prior to it reaching its intended site of action, thereby resulting in poor vaccine efficacy (Chen et al., 1996). Lipid vesicles formed from phospholipids including liposomes can be modulated to increase their stability and acceptability to a wide range of antigens by manipulating the main lipid used within the formulation. Lipids with different chain lengths can be used to alter the phase transition temperatures which can alter the release mechanisms of the entrapped antigen. In addition to altering the phase transition of the vesicles, the change in the hydrophobic tail lengths also influences the molecular packaging configuration of the vesicles. A recent review by Moghaddam et al (2011) on the monolayer studies of liposomes shows that increasing the hydrophobic tail regions of the lipids results in increased pressure being required to distort the lipid monolayer (Moghaddam et al., 2011a). This is ideal when a highly hydrophobic antigen is present as it will have a higher chance of encapsulating more antigen within the bilayer (Uchegbu and Vyas, 1998).

In general lipid based vesicles provide several advantages of antigen delivery where they can be tailored to have desired effects *in vivo*. The application needs to be further enhanced to see the effects in humans, lipid based vesicles provide a significant technological platform however due to the low number of marketed drugs available for

oral delivery the field is not moving at the rate it should be. The current focus is on making vaccines safer by replacing the attenuated pathogens with subunit vaccines such as recombinant proteins or synthetic peptide antigens. These synthetic peptide antigens are referred to as new generation vaccines which are usually non-immunogenic. Lipids themselves are non-immunogenic; however, lipids themselves may undergo physical or chemical transformations over a period of time which may alter stability or formulation performance. Long term and continued exploration of these lipid based formulations will enable an ideal formulation to be doctored at some stage in the future.

1.6.2 Niosomes as vaccine delivery systems

Given the ability of many surfactants to form bilayer vesicles, it is no surprise that such a wide range of components have been used to construct these systems. In the case of non-ionic surfactants, the ability of these molecules to act as an alternative to lipids as building blocks for bilayer vesicles (known as non-ionic surfactant vesicles (NISVs) or niosomes) was first reported by Baillie et al, (1985). These niosomes offer all of the structural attributes of liposomes in terms of their ability to entrap drugs and antigens within the bilayer and/or the aqueous phase, they can be formed in a range of sizes from small unilamellar to large multilamellar structures, and they can be surface modified in a range of ways similar to liposomes (Uchegbu and Florence, 1995). In addition to these standard attributes, niosomes have also been shown to offer additional abilities such as forming polyhedral vesicles in aqueous media (Arunothayanun et al., 1999) and have been suggested to offer greater chemical stability compared with liposomal systems and reduced special handling upon storage (Baillie et al., 1985, Conacher et al., 2001). Furthermore in harsh biological environments (such as within the gastro-intestinal tract) they have been shown to offer greater stability to acidic and enzymatic degradation compared to liposomes (Yoshida et al., 1992, Rentel et al., 1999). However like

liposomes, the formation of niosomes requires the input of energy in forms such as physical agitation or heat where the structure consists of an enclosed aqueous core (Uchegbu and Vyas, 1998).

Niosomes have been widely used in cosmetics and were first patented by L'Oreal in the 1970s with their first use being in 1979 as cosmetic products (Handjani-Vila et al., 1979). Since then, they have been investigated for the delivery of a wide range of drugs e.g. anticancer agents (e.g. Hong et al., 2009, Uchegbu and Duncan, 1997), low solubility drugs (e.g. Mokhtar et al., 2008), antigens (e.g. Murdan et al., 1999, Gupta et al., 2005) and gene therapy (e.g. Huang et al., 2006). An early review by Gregoriadis (1990) has shown that the association of antigen with liposomes improves antigen delivery to the antigen presenting cells (APCs) and this attribute is not restricted to liposomes; niosomes, have also been shown to enhance cell mediated immunity in addition to humoral immunity (Brewer and Alexander, 1992, Brewer and Alexander, 1994).

Whilst niosomes are analogues of liposomes, the surfactants used to formulate niosomes can offer increased chemical stability compared with their phospholipid counterparts and therefore, as mentioned above, maybe more resistant to degradation within the GIT (Conacher et al., 2001). Unfortunately, there are limited studies on the interactions between dendritic cells and niosomes; however, several studies have investigated their adjuvant properties which have been confirmed by *in vivo* studies. As discussed by Uchegbu and Vyas (1998), there are various factors which influence niosomes and their chemical stability. These factors are generally similar to the factors which also influence other particulate delivery systems which include nature of drug, hydration temperature, nature of surfactants, characteristics of additives, size reduction

techniques and the energy added to the niosome vesicles. Niosomes can be modified in terms of drug/antigen loading by altering the nature of the hydrophilic head groups this in turn can increase drug efficiency.

1.6.2.1 Building non-ionic surfactant vesicles (Niosomes, NISV)

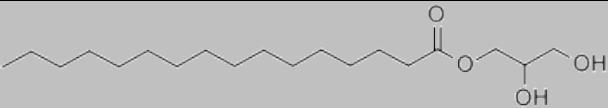
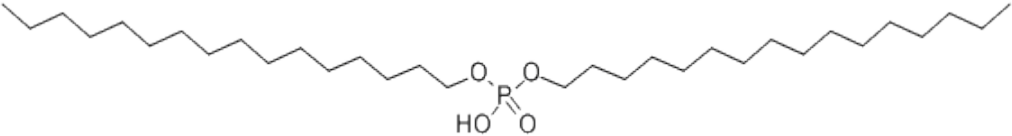
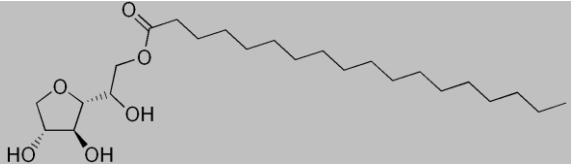
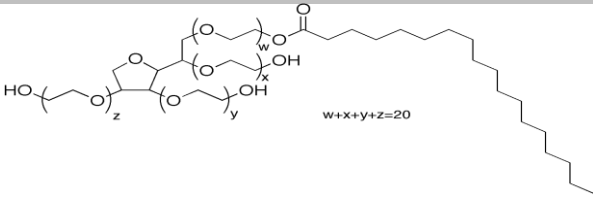
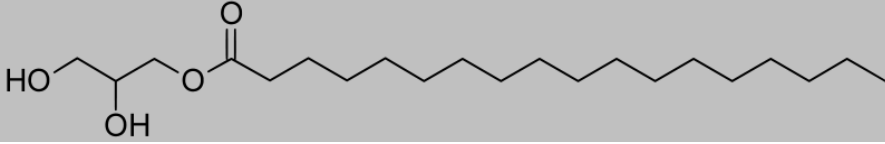
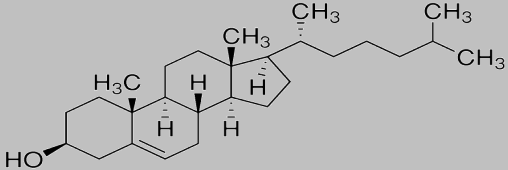
There are a wide range of non-ionic surfactants that can be used to prepare niosomes and examples of the types of surfactants which can be used are given in table 1.1. These surfactants are most commonly tested and formulated into niosomes with the presence of cholesterol due to their chemical stability, resultant sustained release of drugs, low toxicity profile and enhanced permeation when delivering via the skin (Manosroi et al., 2003).

In general, the formation of non-ionic surfactants into bilayer vesicles is dependent on many factors including: temperature, surfactant concentration, electrostatic and electrodynamic interactions of the surfactants within the aqueous phase. Furthermore the molecular attributes of the surfactant is also an important parameter as the type of colloidal or vesicular structure a specific surfactant forms is to a large extent dictated by its molecular shape and the mixture of surfactant combinations used, as this will influence its geometrical packing properties in a given solution environment. The shape of a surfactant may be expressed as its critical packing parameter (CPP) which is defined as:

$$CPP = \frac{v}{a_o l_c}$$

CPP	is the critical packing parameter,
v	is the molecular volume of the hydrophobic part of the lipid,
a _o	is the surface area of the hydrophilic head group,
l _c	is the length of the hydrocarbon chain.

Table 1.1: Properties of some commonly used surfactants in the preparation of niosomes.

Surfactant	Structure	Molecular Weight	Melting point (°C)	T _c (°C)
1-monopalmitoyl-rac-glycerol (MPG)		330.5	71-72	23
Dicetyl Phosphate (DCP)		546	74-75	-
Sorbitan monostearate (span 60)		430.62	54-57	55
Polyethylene glycol sorbitan monostearate (Tween 60)		1309	55-60	40.6
Glyceryl monostearate (GMS)		358.57	58-59	>65
Polyoxyethylene (4) lauryl ether (Brij 30)	$C_{12}H_{25}(OCH_2CH_2)_4OH$	362.5	1.67	<10
Cholesterol		296.65	147-149	-

The CPP value can be used as an indicator to predict geometry of surfactants and the resultant structures they can form as represented in figure 1.3: a CPP value < 0.5 (indicating a large contribution from the hydrophilic head group area) suggests the surfactant forms spherical micelles, a value between $0.5 < \text{CPP} < 1$ forms bilayer vesicles and a CPP > 1 (indicating a large contribution from the hydrophobic group volume) results in inverted micelles (Uchegbu and Vyas, 1998, Biswal et al., 2008, Perrie and Rades, 2009, Israelachvili and Mitchell, 1975, Israelachvili et al., 1977). Therefore, depending upon the type of vesicle required, the CPP gives a good indication on the surfactants that can be used.

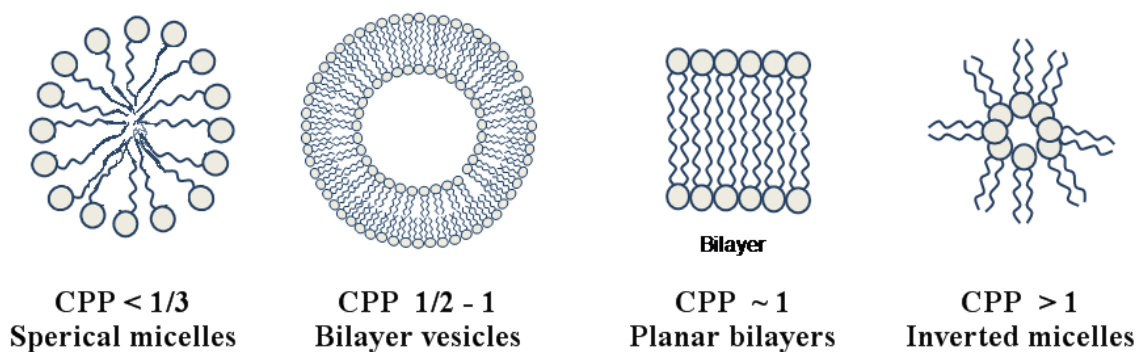


Figure 1.3: The critical packing parameters of lipids and the structures they form.

It is interesting to note that many single chain surfactants have CPP < 0.5 and form micelles, yet these are often used to formulate niosomes; however, not without the addition of additional components. For example, soluble surfactants (such as solulan C-24 and polysorbate 20) with high hydrophilic-lipophilic balance values (HLB) readily form micelles. Yet with the inclusion of cholesterol into the mixture, niosome vesicle structures are formed, thus increasing the physical stability of the vesicles (Uchegbu and Florence, 1995, Manosroi et al., 2003, Carafa et al., 1998). The ability of cholesterol to promote the formation of two-phase bilayer systems, rather than single phase micellar solutions, is related to the overall CPP of the lipid mixture. It is understood that in cases where a mixture of surfactants and additives such as

cholesterol are used to prepare vesicles, the operational CPP values will consider the average of the overall components (Kumar, 1991) and Manosroi et al, (2003) have confirmed that as cholesterol is incorporated within a Tween 61 surfactant mixture, an average CPP value between 0.5 - 1 was obtained hence the formation of bilayer vesicles rather than micelles (Manosroi et al., 2003). Similarly, Tween 20 has a CPP below 0.5; however, when mixed with cholesterol unilamellar vesicles were formed (Carafa et al., 1998) as does with Span 60, which is widely used in the formulation of niosomes (Ning et al., 2005, Uchegbu et al., 1995). This is due to the average CPP of the mixture moving into the range of 0.5-1, hence showing capabilities of forming bilayer vesicles. Due to high phase transition temperatures of Span 60, leakage of drug molecules from the bilayer has been reported (Srinivas et al, 2010); the incorporation of cholesterol has also been shown to stabilise the vesicles by abolishing the phase transition temperature of the vesicles hence avoiding leakage of drug molecules from the bilayer (Srinivas et al., 2010, Uchegbu and Vyas, 1998). It is clear from the thermodynamic consideration that bilayer structures do not exist as such in the absence of water because it is water that provides the driving force for surfactant molecules to assume a bilayer configuration and unlike micelles, the assembly into closed bilayers is rarely spontaneous and usually involves input of energy such as physical agitation or heat (Lasic, 1990). The excess free energy change between water and a hydrophobic environment explains the preference of appropriate surfactants to assemble into bilayer structures, excluding water as much as possible from their hydrophobic regions, in order to achieve the lowest free energy level and consequently the highest stability for the aggregate structure.

Another commonly used non-ionic surfactant, monopalmitoyl-glycerol (MPG) used to formulate niosomes for a range of studies (e.g. Brewer and Alexander, 1992), requires

the inclusion of additional surfactants to support vesicle construction. The addition of cholesterol and dicetyl phosphate to MPG forms stable niosomes and the ratio of these combinations can dictate the physical attributes of these systems. This interplay between vesicle size and cholesterol content has also been noted by Van Hal et al, 1996 who reported a decrease in vesicle size upon increased cholesterol content. Furthermore, cholesterol has the ability to influence the packing density within the bilayer and in turn increase the planarity of the bilayer resulting in uniform bilayers (van Hal et al., 1996, Moghaddam et al., 2011a). In addition, DCP incorporation in niosomes has been proven in several studies, including vaccine delivery to alcohol free niosome gel formulations, to stabilise vesicles against fusion and aggregation where the vesicles obtain their net negative charge (Uchegbu and Florence, 1995, Barakat et al., 2009, Jadon et al., 2009).

1.6.2.3 The addition of cholesterol to niosome formulations

Given the above outlined advantages the addition of cholesterol can offer to a formulation, it is understandable why nearly all current studies investigating niosomes have incorporated cholesterol into the vesicle composition. Indeed when dispersed without the presence of cholesterol, high solubility non-ionic surfactants form micelles and low solubility non-ionic surfactants often form a gel (Yoshioka et al., 1994). However the use of cholesterol not only promotes vesicles formation from a wide range of non-ionic surfactants, its inclusion in both liposome or niosome formulations is known to remove the gel-liquid transition of the surfactant bilayer and hence influences bilayer fluidity (Taylor and Morris, 1995).

The preparation method is a key parameter in the formation of structured bilayers when considering the transition temperature. For example, the hydration temperature determines the overall size and shape of the vesicles formed. In both liposome and

niosome systems the bilayer membrane is an ordered structure and may exist in the gel state, indicating a situation where the alkyl chains are tilted at a slight angle with respect to the plane of the bilayer, or the liquid crystalline state. The difference between these two phases is the degree of order, with the gel state being the most ordered structure than the liquid crystalline state. In the liquid crystalline state there is lateral diffusion of bilayer material whereas in the gel state the alkyl chains are crystallised or otherwise less mobile. For any system, the liquid crystalline state exists at a higher temperature than the gel state. The increase in the temperature (T_c), although yielding an increase in the enthalpy term (ΔH), also results in an increase in entropy (ΔS) and thus a lowering of the free energy (ΔG) of the system occurs and it is the application of heat that is the driving force for this transition (New, 1990). Hence the formation of niosome vesicles should occur above the T_c of the gel to liquid phase transition allowing mobility of the bilayer and hence reordering of surfactants to produce spherical uniform vesicles.

Interestingly, controlling the phase in which the bilayer is in can influence the structure of niosomes: for example not all niosomes are spherical in nature, as is the case with polyhedral niosomes (Arunothayanun et al., 1999). The niosomes in this study were prepared by hydrating thin films of surfactant/lipid blends where they were analysed for morphology and rheological properties. The studies showed that niosomes formed from hexadecyl diglycerol and other polyoxyethylene alkyl esters form a variety of shapes due to the differences in membrane composition. The incorporation of cholesterol results in large spherical vesicles; however, the exclusion of cholesterol resulted in the formation of polyhedral niosomes (Arunothayanun et al., 1999). These polyhedral niosomes were heated above their transition temperature, at which a reversible shape transformation into spherical niosomes occurred indicating that the niosomes go from a

gel state at room temperature to a liquid-crystalline state at their T_c . As a result, the rheological properties of the polyhedral niosomes are more rigid and viscous in comparison to their spherical counterpart structures at the elevated temperatures. Therefore, when formulating niosomes for different routes of administration, characterisation should be carried out to ensure the niosomes are able to effectively perform at their intended site of action.

1.6.3 Bilosomes

Vesicle lysis of lipid based vesicles when administered orally has been the foundation for the development of modified lipid based vesicles which have increased stability to allow the vesicle to reach the site of action and to have increased absorption through the GIT. These modified vesicles are known as bilosomes, which are a relatively new drug delivery system for oral vaccine delivery. Bilosomes intend to protect antigens from the enzymes present in the GIT and they can act as potent immunological adjuvants. Bilosomes incorporate bile salts, such as sodium deoxycholate, into the niosome formulation (Bennett et al., 2009) protecting them from the effects of bile acids within the GIT (Schubert et al., 1983). The aim is to aid vaccine technology by increasing the stability of the carrier thus, preventing premature release of the protein/ antigen via the oral route.

Research suggests (Schubert et al., 1983) that by incorporating bile salts into the vesicles they resist degradation and disruption from the digestive enzymes (Figure 1.4) therefore making the formulation more stable and giving the potential rise for an oral route of delivery for vaccines (Conacher et al., 2001). By preventing premature release, bilosomes deliver the vaccines to the site of action which means the antigen is directly delivered to the mucosal tissue. As a result of the bilosomes delivering the antigen to

the mucosal tissue, smaller concentrations of the antigen can be used to provide an effective response. An advantage of this is that a wide range of antigens can be used, including weaker ones as the delivery is site specific.

Alexander and Brewer (Brewer et al., 2001) first developed and formulated bilosomes by exploiting non-ionic surfactant vesicle (NISV) technology. NISVs resemble liposomes in their structure but possess very low toxicity thus, can function as successful adjuvants which elicit immune responses after parenteral administration. Bilosomes use NISVs as their structure but with the incorporation of bile salts as part of the vesicle structure. Recent studies using bilosomes incorporating several antigens have proven to be successful in animals such as the A/panama (Mann et al., 2004), tetanus toxoid (Mann et al., 2006) and hepatitis B (Shukla et al., 2008). These studies on the various antigens have shown significant antibody titres and have also shown increased antibody production, lower temperatures and reduced symptoms in relation to IM injections when dosing with influenza haemagglutinin entrapped bilosomes (Bennett et al., 2009). Bile salt incorporation within the vesicles, stabilise and protect the vesicles from the GIT (Figure 1.4) and other enzymes present in the body allowing bilosomes to be used orally as vaccine drug delivery system. In the bilosome studies by Mann et al, (2006), Bennet et al, (2009), Mann et al, (2004) and Mann et al, (2009) the anionic lipid dicetyl phosphate (DCP), is used in the formulation of bilosomes. These studies all show that, by orally administering the bilosome vesicles with the antigen entrapped, a mucosal immune response is elicited with specific IgA production increased from the mucosal cells in the small intestines is initiated.



A



B

Figure 1.4: Representation of vesicles with and without bile salts. Present are two pathways where **A)** is a normal lipid vesicle which is disrupted by the intestinal bile salts, causing degradation and vesicle lysis and pathway **B)** where the lipid vesicles have bile salt incorporated into the structure prior to gastrointestinal exposure shows membrane stability when in the presence of intestinal bile salts.

The production of IgA levels suggests that the oral immune response has been successfully initiated (Figure 1.1). The incorporation of the DCP lipid within the formulation results in the vesicles having a highly negative surface charge which could potentially be the reason for such increased levels of immunity achieved from orally dosing the bilosome vesicles. These studies confirm previous work by Tabata and Ikada (1988) where they showed that the peak uptake of particles occurs with a zeta potential of -70 mV in the epithelial cells (Tabata and Ikada, 1988). This is also in line with studies by Norris et al, (1998) and Eldridge et al, (1990) where they also found that the uptake of negatively charged particulates is favoured (Norris et al., 1998, Eldridge et al., 1990). In addition, the work carried out by Shakweh and co-workers (2005) suggests that negatively charged or neutral particles in mice have a greater affinity for the Peyer's patches than positively charged particles (Shakweh et al., 2005). As a result, various studies carried out on mucosal particulate uptake all stem down to the presence of a negative surface charge. This allows uptake by the M cells within the Peyer's patches and trigger the dendritic cells and initiate the immune response.

1.7 Virosomes

Virosome vesicles are reconstituted viral envelopes which are made of unilamellar phospholipid bilayers which incorporate virus derived cell binding and fusion properties. The key to virosomes are that they are absent in the genetic material of the virus which is the 'danger' part of the virus (Copland et al., 2005). Virosomes, as they are formed by the extraction of glycoproteins and phospholipids from virus particles they can be engineered and tailored to incorporate antigenic material. This antigenic material can be incorporated into the lipophilic or hydrophilic regions of the virosome depending upon the antigen solubility. Virosomes are similar to liposomes; however, the presence of haemagglutinin allows the virosome mechanism of action to vary where

it can deliver the contents directly to the cytoplasmic compartment. Currently the only licensed virosome formulations are parenteral formulations taken intramuscularly the products are Epaxal (Hepatitis A) and Inflexal V (Influenza seasonal) which is patented and developed by Berna Biotech Switzerland (Copland et al., 2005).

Virosomes although they are delivered IM provide a platform which can be adapted to many vaccines where lipids, adjuvants, combination of antigens and other materials such as bioadhesives can be added to the viral membrane and possibly administered orally to animals to test the efficacy. Potentially, studies may modify the virosome surface to be exposed to mucosal surfaces; however, this will need to be monitored carefully as the delivery system will be highly immunogenic and as a result no immune response should be created to the delivery system.

1.8 Mucosal adjuvants

A range of immunostimulatory agents are available such as monophosphoryl lipid A, *Quillaja saponaria* (Quil-A), CpG oligodeoxynucleotides, trehalose 6,6-dibhenate (TDB). Currently, the most potent mucosal adjuvants which are studied for immunisation are the cholera toxin (CT) and the heat labile enterotoxin *E.coli* (LT) (Tamura and Kurata, 2000, Holmgren et al., 2003, Plant and Williams, 2004). These mucosal adjuvants provide long lasting immune responses either systemic or mucosal in turn, allowing the body to induce immunity at low antigen concentrations and doses (Holmgren and Czerkinsky, 1992, Rappuoli et al., 1999). The major drawback of these two mucosal adjuvants are that they are extremely toxic hence are not in human clinical trials. As a result, there is research being carried out on acceptable derivatives of these toxins with reduced toxicity but retaining the adjuvant activity for use in humans. One of these products includes a non-toxic recombinantly produced Cholera toxin B subunit

(CTB) which promotes mucosal immunity sIgA which is important in the elucidation of an oral immune response. In addition to mucosal immunity, the CTB also provides anti-inflammatory tolerance to self antigens (Holmgren and Czerkinsky, 2005, Stanford et al., 2004). Other examples of current mucosal adjuvant derivatives include detoxified mutants of the LT where the active toxic subunit has been modified to remove the toxic components. This has resulted in the loss of adjuvanticity however, studies show that there are a few proteins with significant adjuvanticity even without the presence of the detectable toxic component when administered intranasally (Pizza et al., 2001). A recent review by Baudner and O'Hagan (2010) and studies by Holmgren and Czerkinsky (2005) show different mucosal adjuvants and their effectiveness; however, the majority of studies focus on nasal administration (Holmgren and Czerkinsky, 2005). A promising approach has been identified where hybrid molecules of the cholera toxin subunit has been linked to specific APC (CTA1-DD) from a bacteria *Staphylococcus aureus* (Lycke, 2004). The incorporation of this CTA1-DD and antigen into ISCOM vesicles may provide oral usage possibilities by maintaining adjuvant effectiveness (Holmgren and Czerkinsky, 2005).

Novasome[™] adjuvants, also referred to as non-phospholipid liposomes (Chambers et al., 2004) are multicomponent adjuvant systems made up of dioxyethylene cetyl ether, cholesterol, and oleic acid have been licensed for veterinary application (for the immunisation of fowl against Newcastle virus disease and avian rheovirus). The Novasome[™] technology, made with glycerol monostearate and butyl alcohol and included the potent adjuvant monophosphoryl lipid A (Chambers et al., 2004), was shown to offer protection of guinea pigs against aerosol challenge with virulent *M. bovis*.

Mucosal adjuvants show promising data in terms of eliciting an immune response by increasing the immunity to low concentration antigens however, due to the toxicity of the adjuvants they have been rendered unsafe to use for human clinical trials. Mucosal adjuvants essentially if formulated to show minimal toxicity can be administered and used in combination with other particulate delivery systems to enhance mucosal immune responses therefore, reducing the requirement for multiple dosing or high concentrations of antigen use.

1.9 Characterisation of niosomes – commonly measured parameters

To achieve effective sub-unit vaccine delivery or drug delivery via the use of niosomes, it is essential to study the physico-chemical characteristics which will determine the stability and efficacy of the delivery system. In general, there are various methods employed to characterise delivery systems which include vesicle size, suspension pH, vesicle zeta potential, entrapment loading studies and microscopy studies such as freeze fracture, transmission electron microscopy or basic light microscopy. It is important to ensure that the niosomes produced and tested whether *in vitro* or *in vivo* have the same characteristics in terms of charge, vesicle size, and amount of antigen or drug entrapped as differences could lead to differences in efficacy and results obtained.

1.9.1 Antigen entrapment within vesicles

Entrapment efficiency is obviously an important parameter as it influences surfactant/antigen ratio and total amounts of antigens needed. It can be measured using a range of techniques including: separating the unentrapped drug or antigen e.g. by centrifugation (Brewer and Alexander, 1992), dialysis (Baillie et al., 1985) or gel filtration (Uchegbu and Florence, 1995). For protein and peptide-based antigen assays such as the ninhydrin assay can be used to measure entrapment of peptides and proteins within niosomes as this assay eliminates any interference of lipids or surfactants when

compared to other assays such as the BCA assay (Brewer et al., 1995). In some cases the Lowry method for protein concentrations are used, which works on the general principle under alkaline conditions where the divalent copper ion reacts with peptide bonds hence forming a complex in which it is reduced to a monovalent ion. This monovalent copper ion, along with the other radical groups of the reagents, react with a Folin reagent resulting in an unstable product which is reduced to tungsten blue. The limitations of this method are that for the reaction to occur a pH of 9-11 needs to be maintained and that certain buffers, lipids and drugs may interfere with the assay hence appropriate filtration and dilutions are needed before running the assay (Lowry et al., 1951). Due to the limitations of the Lowry method, a simpler and less sensitive to interference assay is the Bradford assay. The Bradford assay is analysed at a wavelength of 595 nm as this becomes the lambda max for the reagent Coomassie brilliant blue G-250 when protein binding occurs. The colour change is observed due to the stabilising effect of the anionic dye by hydrophobic and ionic interactions of the reaction (Kruger, 2002).

1.9.2 Vesicle diameter and morphology

The vesicle size and polydispersity of a niosome suspension can be determined using various techniques including: e.g. light microscopy, freeze fracture electron microscopy and laser diffraction. Laser diffraction is commonly used in the analysis of particle size for suspensions, aerosols, emulsions and other pharmaceutical products. Of the physical attributes, particle size and polydispersity are often seen as key factors that may affect product stability and *in vivo* efficacy. The basic principle of laser diffraction is that a laser which supplies the light is passed through the suspension and the interactions of the light with the particles (diffraction) is measured. Generally, the laser beam runs parallel to the laser diffraction sensors and the intensity of the diffracted laser from each

particle which passes through a Fourier lens is measured by a detector. The intensity of the diffraction is recorded on the photo-detector which establishes the particle size. Within the studies a wet dispersion system will be used with a Quartz cuvette which creates a dynamic event rather than a static measurement and any simultaneous diffraction on more than one particle will result in a superposition of the diffraction patterns of the individual particles where an average will be taken.

As already noted, particle size is a critical factor in determining the fate of orally delivered particulates both, in terms of their uptake and potentially the type of response the systems potentiate. Whilst three possible routes of GI uptake of small particles (intracellular uptake by enterocytes, intercellular/paracellular transfer and uptake via the M cells of the Peyer's patch) have previously been considered, it is now mainly agreed that particulate uptake in mammals is chiefly via the M cells of Peyer's patches (Lavelle et al., 1995).

When orally administering vaccines the size of the carrier systems are crucial for determining uptake and retention at the immuno-competent cells within the gut which are known as Peyer's patches. Studies by Eldridge et al. (1990) on vesicle size uptake in the Peyer's patches show that uptake is dependent on both the vesicle size and hydrophobicity (Eldridge et al., 1990). The results from their experiments indicate that a vesicle size of 10 μm or less are taken up by the Peyer's patches; however, particles with a size less than 5 μm can traffic to other tissue cells by a process of lymphoid drainage. Therefore, the vesicle size plays a crucial role when designing vaccine carrier systems to successfully allow the vaccine and the carrier to reach and retain at the target site to allow them to exert their therapeutic effect. In addition to laser diffraction, it is important to confirm the shape and structure of the niosomes and microscopy techniques can be used to consider this.

A review by Bibi et al, discusses the advantages and limitations of specific microscopy techniques to analyse vesicles (Bibi et al., 2011). Although there are several microscopy techniques available for the image analysis of vesicles, techniques such as light microscopy offers basic conformation of morphology and size of the vesicles. However, when examining vesicles in greater detail, advanced microscopy techniques are available such as the various transmission electron microscopy (TEM) techniques which include, negative stain TEM, Cryo-TEM and freeze fracture TEM. In particular the use of freeze-fracture can give greater detail on both the size and number of bilayers the niosomes have; however general light microscopy can offer a good overview of heterogenicity.

1.9.3 Surface charge

In terms of characterising niosomes, the zeta potential is the simplest way to measure surface charge of vesicles and gives a representation of the stability of a formulation. A highly positive or negative zeta potential can imply that the vesicle suspension is more likely to resist aggregation (Freitas and Müller, 1998, González-Rodríguez and Rabasco, 2011). Zeta potential is a function of the vesicle surface charge which is calculated at the surface interface and the nature of the surrounding medium. The Zeta potential is the nearest experimental approximation of surface potential in millivolts (mV), as both the surface potential and stern potential are difficult to measure. When an electric current is passed through each particle, the rate and extent of ion movement within the double layer and the surrounding medium can be used to determine the zeta potential. Hence, the zeta potential is the potential at the surface of shear which is measured by electrophoresis and reflects the effective charge on the vesicles and the repulsion between them, in turn giving an insight into the stability and flocculation of the suspension (Florence and Attwood, 2006).

In terms of surface charge, anionic, cationic and neutral systems have been used as oral vaccine delivery systems. In most cases PLGA microspheres, unless modified, will have an anionic charge due to the presence of the carboxylic groups, and as already noted these particulates are taken up after oral administration (Shakweh et al., 2005). The bilosome formulations investigated are all highly negatively charged as they are formulated from bile salts, non-ionic surfactant, cholesterol and the anionic surfactant dicetyl phosphate (DCP), generally at around a 10 % molar content. Based in fluorescent microscopy studies, anionic liposomes formulated using phosphatidyl serine (PS) were also shown to have improved uptake by Peyer's Patches compared to liposomes formulated without the inclusion of PS (Tomizawa et al., 1993, Aramaki et al., 1993). Norris et al, (1998) and Eldridge et al, (1990) also found that the uptake of negatively charged particulates was favoured (Norris et al., 1998, Eldridge et al., 1990). The work carried out by Shakweh and co-workers (2005) also compared the binding and uptake of differently charged PLGA microsphere formulations and noted that whilst the binding to Peyer's Patch free tissue was similar for all microspheres over 48 h, the uptake by Peyer's patches was notably higher for negatively charged or neutral particles in mice positively charged particles (Shakweh et al., 2005).

However cationic formulations (e.g. employing cationic lipids or chitosan), often employed for DNA delivery, have also been shown to be effective oral vaccine adjuvants. For example, a cationic liposomal oral mycobacterium DNA vaccine formulated using LipofectamineTM 2000 (Invitrogen Corporation, USA) was shown to be effective in protecting and delivering plasmid DNA via the oral route to the epithelium, M cells, DCs and Peyer's patches of the small intestine of mice, and subsequently they were able to promote expression of the encoded Ag85A antigen at these sites. By doing so the cationic liposome formulation was able to induce higher

specific mucosal cellular and humoral immune responses compared to DNA alone (Wang et al., 2010). The use of oral DNA vaccines using cationic particulates is also being extensively investigated for vaccination of fish (Rajesh Kumar et al., 2008, Ning et al., 2009, de las Heras et al., 2010).

Whilst considering these surface characteristics, it should be kept in context that the surface charge (commonly considered by measuring the zeta potential) is measured generally in a buffer or water, and not in the various environments of the GIT. Given the changes in pH, the presence of salts, enzymes and other moieties it would not be unsurprising to consider the surface characteristics of these particulates will change. Indeed studying the zeta potential of cationic liposomes was shown to be dramatically different (~ 40 mV vs ~15 mV) depending if the liposomes were in simulated gastric or intestinal media respectively (Perrie et al., 2002). Whilst changes in zeta potential of vesicles, due to changes in pH, are often reversible, such changes may impact on antigen loading which relies on electrostatic interactions.

1.9.4 Hydrophobicity

Further to surface charge of vesicles, the hydrophobicity/hydrophilicity of particles can play an important role in vesicle delivery and uptake. For example, liposome uptake within the body can be influenced by the presence of a polyethylene glycol coating on the vesicle surface which modifies the surface hydrophilicity and in return reduces opsonisation and macrophage recognition (Allen et al., 1995). By reducing opsonisation, the liposomes increase their circulation time within the body however for a vaccine delivery system this may be counter-productive. As a result the hydrophobicity of the vesicles should be taken into consideration when optimising bilayer vesicles for adjuvant activity.

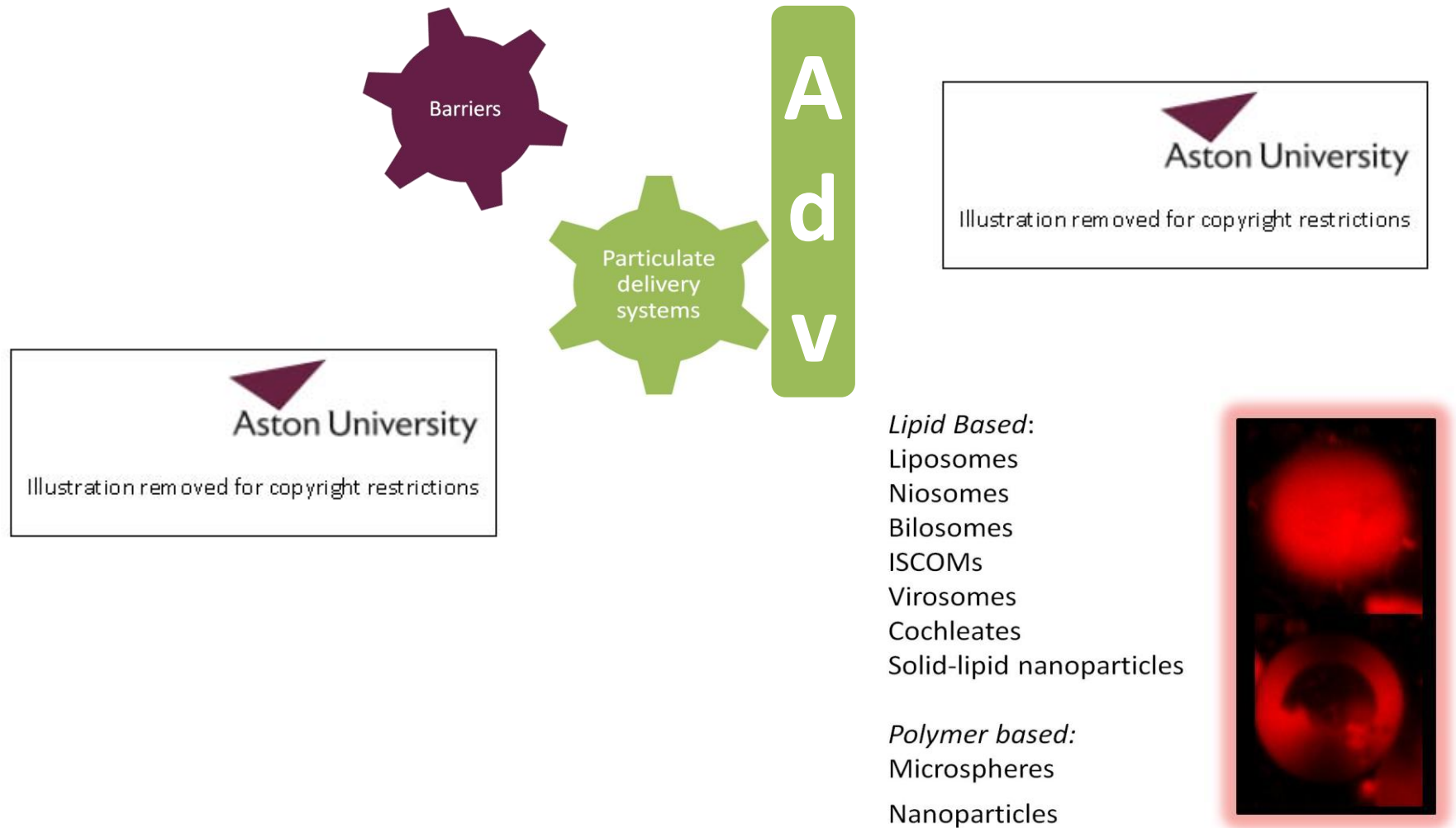


Figure 1.5: Summary of barriers and particulate delivery systems to overcome these barriers.

1.10 Aims and Objectives

Over the decade it has become apparent that more research is needed to develop mucosal vaccines as an alternative to the current needle based vaccines due to the increased pressure of regulations and immunisation strategies. With greater understanding of the mucosal immune response, and the advantages of the immunity achieved via the mucosal route, interesting research is being carried out on the various oral delivery systems to achieving an ideal formulation. Although there are a few oral vaccines on market such as the OPV for human use, it is essential to develop a broader range of mucosal vaccines by using particulate delivery systems which protect the antigens and supply them to the site of action where an immune response can be initiated. The use of effective adjuvants, either in combination, or formulated within the particulate delivery system will promote the mucosal immune response towards the desired effect.

Several particulate delivery systems and mucosal adjuvants have been developed and are showing promising data, their effectiveness and usefulness is yet to be established in humans. The push towards particulate delivery systems stem from the safety issues which arise when administering live or attenuated vaccines as reversion to the virulence form can take place. The use of inactivated vaccines often fail to initiate an immune response where subunit vaccines are generally weak immunogens and are subject to degradation before reaching the site of action. As a result delivery systems which protect the subunit antigen from the external milieu of the body need to be formulated which can incorporate adjuvants exerting its desired effect on the mucosal immune system. Delivery systems include the use of lipid based vesicles (liposomes, niosomes, bilosomes, virosomes etc), micelles, ISCOMs, nanoparticles and microspheres where

they can be manufactured using lipids, polymers, proteins and in some cases metals. The carrier system must not be affected by the type of antigen or be influenced by the payload of the antigen. The major advantage of particulate delivery systems is the diverse range of materials which can be used to produce the particulates and how they can be tailored and modified to give specific release profiles and protection of antigen or drugs.

As a result, the aim of this research was to design and produce a delivery system with desirable characteristics of size, charge, hydrophobicity and antigen retention to enable successful delivery of subunit vaccines when administered intramuscularly or administered to the oral mucosa safely and efficiently. In order to accomplish this, the objectives were to:

- design a suitable carrier system with optimal characteristics for size, charge and antigen entrapment by the use of Design of Experiments and various microscopy techniques.
- understand surfactant structure interactions and molecular packaging by the use of Langmuir monolayer studies and the thermodynamics of the surfactants and vesicles with the use of Thermogravimetric (TGA) and Differential Scanning Calorimetry (DSC) analysis.
- evaluate the oral biodistribution of the niosome vesicles and the associated antigen to determine the physicochemical characteristics with the uptake by the Peyer's patches to determine immunological function.
- compare the immunogenicity and thermostability of the niosome vesicles against a previously published method of manufacture of niosome vesicles.
- develop an alternative solid dosage format in the form of tablets and capsules for the vaccine carrier systems.

Chapter 2

Optimisation of non-ionic surfactant vesicles for oral vaccine delivery

Publications related to this Chapter:

Wilkhu, J. S., S. E. McNeil, Anderson, D.E., Perrie, Y. (2013). "Characterization and optimization of bilosomes for oral vaccine delivery." Journal of Drug Targeting **21**(3): 291-299.

2.1 Introduction

2.1.1 Overview

An effective vaccine is one which prevents infection and disease through its capability to elicit specific immune responses and hence provide protective immunity (Bramwell and Perrie, 2005). However, ease of use is also a key consideration for global vaccination campaigns. In this respect, oral vaccines offer significant benefits over traditional vaccines due to their ease of administration, improved patient compliance (due to their needle-free format), potentially enhanced mucosal immunity, and strong resistance against many pathogens. Furthermore due to its non-invasive nature and ease of access, the oral route can circumvent the need for trained personnel to administer the vaccine. Unfortunately, the ability of many vaccines to withstand the harsh journey through the gastrointestinal tract and to be effectively taken up in the appropriate target site is limited (Wilkhut et al., 2011).

To address this barrier, delivery systems can be employed. For example, lipid based vesicles such as liposomes or niosomes can be used as effective carriers of macromolecules (Bramwell and Perrie, 2006). Yet, many of these constructs can be destabilised after exposure to intestinal bile salts which have the potential to cause the membrane deformation and vesicle lysis, resulting in the release of macromolecules from the vesicle prior to its intended site of action. In response to this, a range of modified lipid based vesicles, which have increased stability and increased absorption through the GIT, have been developed. Such vesicles include bilosomes, which have been shown to protect antigens from the enzymes present in the GIT and act as potent immunological adjuvants (Mann et al., 2004). This has been attributed to the incorporation of bile salts into the vesicles which increases resistance to degradation

and disruption by digestive enzymes (Schubert et al., 1983) allowing the vaccine to reach the target site, the M cells located in the Peyer's patches (Schmucker et al., 1996). The ability of bilosomes to act as potential carriers for oral vaccines has been established by e.g. Conacher et al, (2001) who demonstrated that by including bile salts in the vesicle constructs, the vesicles could prevent antigen degradation within the highly acidic conditions of the stomach and improve oral vaccine efficacy (Norris et al., 1998, Conacher et al., 2001). Similar studies have also confirmed this with a range of antigens e.g. tetanus toxoid (Mann et al., 2006), the A/panama (Mann et al., 2004), and hepatitis B (Shukla et al., 2010). These studies all show that, by orally administering the bilosome vesicles with entrapped antigen, a mucosal immune response is elicited with specific IgA production increased. Therefore, bilosomes can aid vaccine technology by increasing the stability of the vaccine during transit through the GIT, preventing premature release of the antigen during oral transit (Wilkhut et al., 2011). However, from these studies several potential controlling parameters for the design of bilosome constructs become apparent: vesicle size is vital as this determines the end location of the vaccine when administered, and the surface charge of the vesicles (which is measured via the zeta potential, and influenced by the suspension pH) is also important when determining the stability and absorption of the vaccine (Norris et al., 1998).

2.1.2 Design of experiments (DoE)

DoE can be used to plan and conduct experiments in a random order and be used to extract the utmost information from the data obtained. The aim of designing an experiment through MODDE was to gather data in the fewest number of experimental runs, therefore, saving time and resources. In general, MODDE (Umetrics, Sweden) varies the factors in the experiment simultaneously over the planned experiments with

the aim to analyse the data using a mathematical model which the software generates. Using the model, the data can then be interpreted and predictions can be made which will result in the optimisation of the experiment parameters.

Within this study all of the formulations to be tested were calculated using MODDE and each was prepared and was analysed for suspension pH, vesicle size and vesicle zeta potential. The study undertaken in this chapter involves the use of DoE, where bilosomes were produced with and without peptides to see if there are any changes to the responses upon the addition of peptides to the bilosomes. To do this an experimental design was planned out and two copies of the design were used where one was for an empty bilosome study and the other for a study with peptides. The bilosomes were produced as stated in the method in section 2.2.3.

2.1.3 Aims and objectives

In the formulation of bilosomes, a range of formulations have been considered including compositions comprise of monopalmitoyl glycerol: cholesterol : dicetyl phosphate (5:4:1 molar ratio) and bile salt or sorbitan tristearate : cholesterol : dicetyl phosphate (7:4:1) with bile salts (Mann et al., 2004, Shukla et al., 2008). As a result, this study will use MPG as the main surfactant and will be kept constant at a molar ratio of 5 as this is an important component for the formulation of the bilosomes. The major components that were varied in this experiment were cholesterol, DCP and bile salt as represented within the design space in figure 2.1. This experiment has used the capabilities of MODDE to design an experiment for the formulation of bilosomes with the outcome of potentially selecting an optimised formulation. Therefore within this study the aim was to undertake a systematic investigation into the effect of bilosome

composition on their physico-chemical characteristics and then to test the optimum formulation for efficacy. This will be carried out by:

- Using Design of Experiments, a d-optimal factorial design, was used to plan and conduct experiments in a random order and used to extract the utmost information from the data obtained. The relationship between the factors influencing the bilosome composition and physiological and biological outcomes was investigated by optimising for vesicle size, zeta potential, pH and antigen loading for their ability to deliver antigen for Influenza. Using the model the data can then be interpreted and predictions can be made which will result in the optimisation of the experiment parameters.
- Validating the results from the DoE study by an extra series of experiments and then use the optimised formulation, and test for efficacy in a ferret animal model.

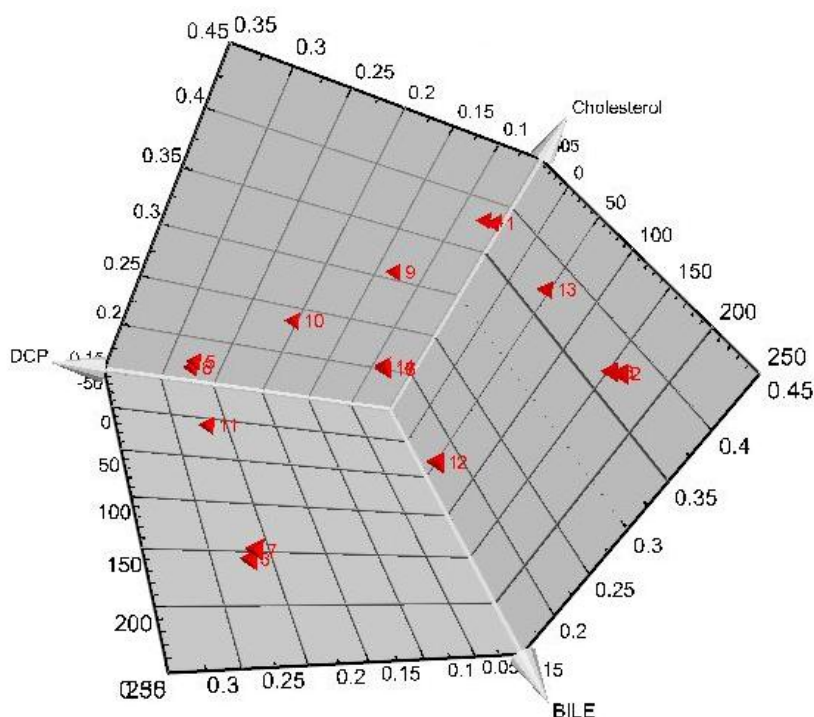


Figure 2.1: Design space for experimental study showing variation in DCP, cholesterol and bile salt.

2.2 Materials and Methods

2.2.1 Materials

A range of non-ionic surfactant based bilayer vesicles were prepared by high shear homogenisation and analysed using several materials and methods. The surfactants used in the study were Monopalmitoyl glycerol (MPG) (Larodan Labs, Sweden), Synthecol (Chol) (Sigma Aldrich, UK) and Dicetyl phosphate (DCP) (Sigma Aldrich, UK). The aqueous buffer sodium bicarbonate (Sigma Aldrich, UK) was buffered to pH 7.6 using a 1 M sodium hydroxide solution (Sigma Aldrich, UK). The peptide antigens used were purchased from Cs Bio (California, USA); Glacial acetic acid was purchased from BDH, Isopropanol (propan-2-ol) from EMD and the 2 % Ninhydrin Reagent was purchased from Sigma.

2.2.2 Preparation of peptide mixture

The peptides used throughout the experiments were dissolved in a mixture of ratios of the 5 peptides which were the combination of epitopes from Influenza A Hemagglutinin H3 and H1, Influenza B Hemagglutinin and Influenza A nucleoprotein subtypes; (i) INF-H1-88-S1, (ii) INF-HA-1-V1(C3S), (iii) INF-NP-1-V1, (iv) 02-HB-01-S1-01 and (v) 02-HB-01-S2-01. The ratios used were (i) 2 mg, (ii) 2 mg, (iii) 2 mg, (iv) 1 mg and (v) 1 mg in 0.8 mL of double distilled water with a final concentration of 10 mg/mL in a siliconised tube. The peptide solution was then vortexed for 4 minutes and then spun down for 2 minutes at 6,000 x g.

2.2.3 Bilosome preparation

A paraffin oil bath was set up at 120 °C and alongside a water bath (Grant, VWR) at 50 °C. Two 25 mM sodium bicarbonate buffers were made up with one at a pH of 7.6 and the other with a pH of 9.7. After the buffers were prepared, the bile salt solution was

prepared by making a 100 mM solution using the freshly made 25mM sodium bicarbonate buffer of pH 9.7.

The 5:4:1 molar ratio of the lipids 135 mg of MPG, 127.5 mg of Chol and 45 mg of DCP were weighed and placed in a 25 mL flat bottom glass beaker (Pyrex, UK) ensuring no powder sticks to the sides of the glass beaker. The lipids were melted by heating at 120 °C for 10 minutes. Once the lipids were set to melt the peptide solution prepared in section 2.2.2 was pre-incubated for 5 minutes at 30 °C.

While maintaining the melted lipid solution at 120 °C, an emulsion was created by the addition of 5.2 mL of the 25 mM sodium bicarbonate pH 7.6 buffer (preheated to 50 °C) and immediately homogenised (Silverson L4RT, UK) for 2 minutes at 8000 rpm. While still homogenising 0.55 mL of 100 mM bile salt in 25 mM sodium bicarbonate buffer pH 9.7 was added and homogenised further for 3 minutes. After the 3 minutes homogenising 0.28 mL of the pre-incubated peptide solution was added to the beaker containing the suspension and was homogenised for a further 5 minutes. Once homogenisation had finished the bilosome suspension was allowed to cool to room temperature and was then left for 2 hours in an incubator/shaker at 220 rpm.

2.2.4 Characterisation of size, charge and pH

The vesicle size distribution was determined using laser diffraction on a sympatec 2005 (Helos/BF) cuvette analyser. 20 µL of the bilosome suspension was diluted into the cuvette with 40 mL double distilled water. The zeta potential which is an indirect measurement of the vesicle surface charge, was measured in 1.5 mL double distilled water at 25 °C on a Zeta Plus Brookhaven Instrument. 15 µL of the niosome suspension was mixed in 1.5 mL double distilled water and then analysed. The pH studies were

determined using a pH meter (Mettler Toledo), the size of the bilosomes were determined using a sympatec 2005 (Helos/BF) analyser.

2.2.5 Centrifugation optimisation

For quantification of peptide entrapment, ultra-centrifugation of the bilosome formulations was required to isolate peptide-entrapped bilosomes, from non-incorporated antigen. To achieve this 300 μ L aliquots of sample (Optimised in Figure 2.2) was diluted in a Beckman 3.9 mL Polo-allomer tube and filled up to the neck with double distilled water. The tubes were carefully heat sealed ensuring that there were no air bubbles present within the tube. The tubes were then placed accordingly in the TLN-100 rotor and centrifuged at 354,000 X g for 45 minutes at 4 °C with the acceleration set at 8 (Slowest) and the deceleration at coast. When centrifugation was complete, the tubes were opened and the supernatant removed. The sample was resuspended and the centrifuged state repeated. After the second 'wash' the peptide-loaded bilosomes were then re-suspended with 100-200 μ L of appropriate buffer and transferred to a clean/labelled 1.5 mL micro-centrifuge tube.

2.2.6 Determination of peptide loading in vesicles

The peptide loading was determined on the pellets produced after centrifugation as section 2.2.4 the using the modified Ninhydrin assay (Figure 2.3) at a wavelength of 560 nM (Brewer et al., 1995). Following centrifugation, supernatant was removed with a pipette and vesicles re-suspended. Then 100 μ L of the sample into a 1.5 mL polypropylene micro-centrifuge tube and the samples were then dried at 110 °C on a digital heat block (VWR). When dry, the samples were hydrolysed by adding 150 μ L of 13.5 M NaOH to each tube.

The tubes were then heated at 110 °C for 20 minutes and then cooled to room temperature. 250 µL glacial acetic acid (Fisher Scientific, UK) was added to the samples to neutralise the NaOH and 500 µL ninhydrin (Sigma Aldrich, UK) solution was added to each tube, vigorously vortexed and then placed on the digital heat block for 20 minutes at 110 °C. 250 µL aliquots of the samples from these tubes were transferred to fresh micro-centrifuge tubes containing 750 µL of propan-2-ol: water (Fisher Scientific, UK) (1:1 in v/v) and vigorously vortexed. The 1 mL sample was transferred into a semi-micro cuvette and the absorbance was read and recorded at a wavelength of 560 nm on a Jenway 6405 UV/Vis spectrophotometer. The amount of peptide present (µg/mL) is determined from a standard curve which is also made at the same time as carrying out the ninhydrin assay of the samples.

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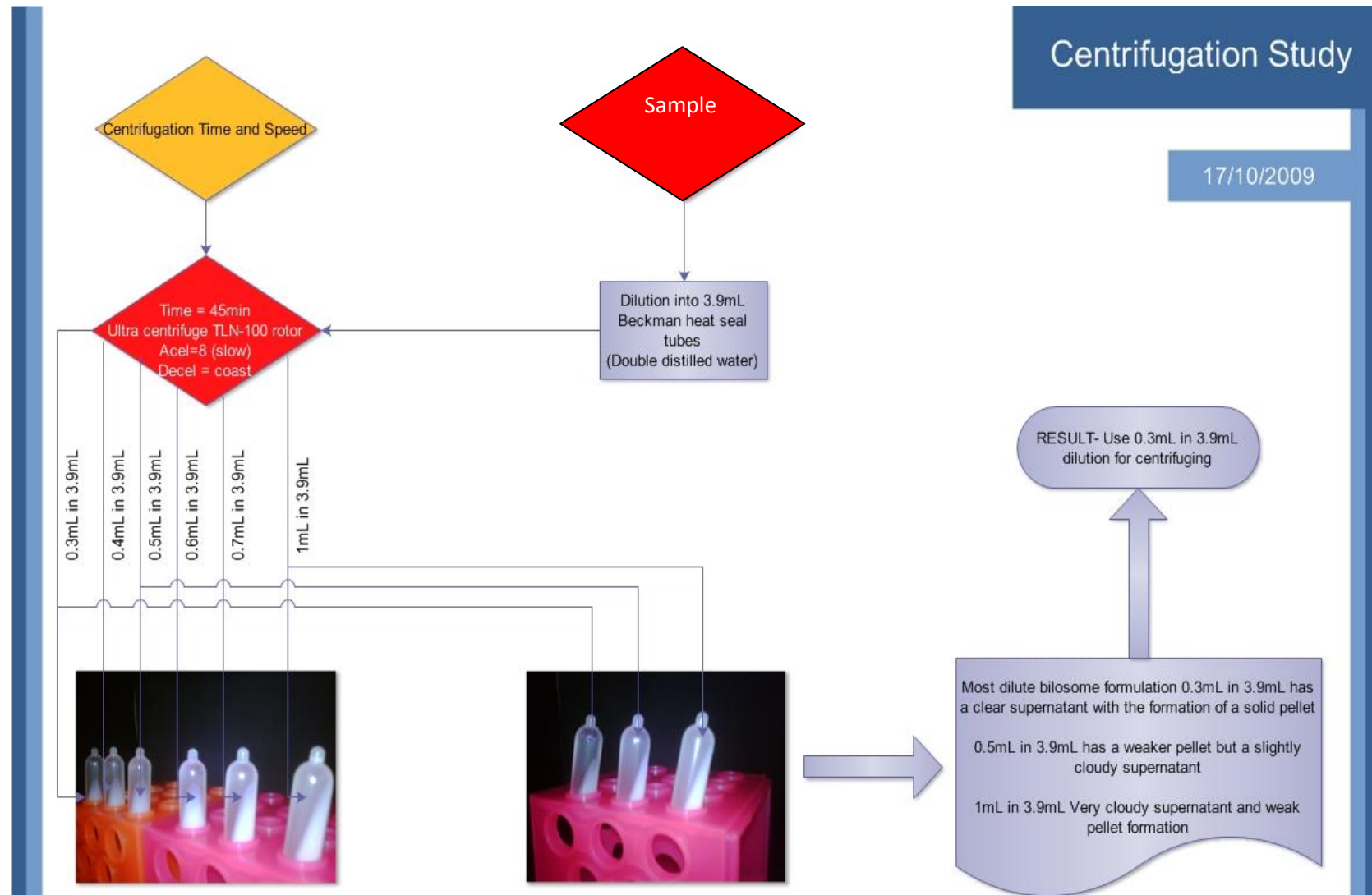


Figure 2.2: Optimisation of centrifugation cycle by optimising sample volume.

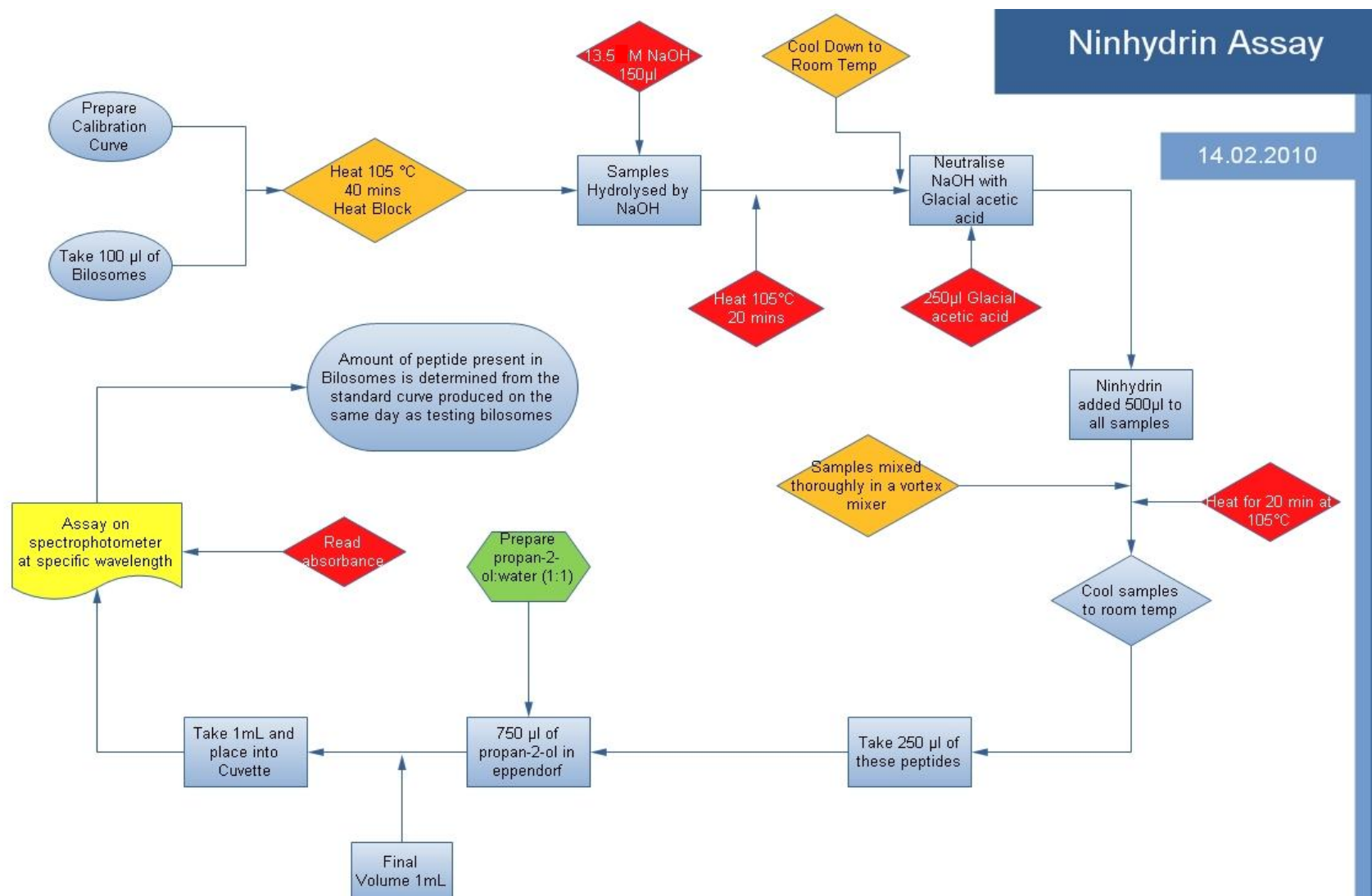


Figure 2.3: Ninhydrin Protocol for determination of antigen concentration within vesicles.

2.3 Results and Discussion

2.3.1 Design of Experiments (DoE) analysis

An experimental design consisting of 3 surfactants and bile salt as the factors was generated with the responses to test being pH, vesicle size, zeta potential and peptide entrapment where the MPG lipid was always kept constant at a ratio of 5 and the cholesterol, DCP and bile salt was varied (Table 2.1). The experimental design consisted of three centre points to form the control for the experiment, by appearing in a random order, with the same molar ratios for each of the factors. The centre points were calculated by taking the average of each factor for all the experimental runs and in turn will determine the accuracy and reproducibility of the experimental model that is calculated.

Table 2.1 shows that the MPG ratio was always kept constant at 0.5 and the varying factors were the bile, cholesterol and the DCP also ratios (Ratios are in fractions due to MODDE set up). Runs 5, 14 and 16 have the same formulation ratios and are the centre points for this experiment and the run order shows the order in which to conduct the experiment to introduce randomness and reproducibility. The results were inputted into the table generated from MODDE showing pH, vesicle size and zeta potential. All responses were taken in triplicate and a standard deviation where $n=3$ (A= Empty vesicles, B= Peptide loaded vesicles).

The overall data (Figure 2.4) show three significant variables which are highlighted from the results of the design of experiments study which involved cholesterol, DCP and bile salt. In particular the DCP and cholesterol lipids together have shown to have a significant impact on the pH and zeta potential with the p -value being less than 0.05 (Table 2.2).

Table 2.1: Experimental plan for the composition of bilosomes using MODDE. The table is generated from MODDE and shows the ratios of each factor to be used into the formulation. **A)** Empty vesicles, **B)** Peptide loaded vesicles.

A

Exp No	Exp Name	Run Order	Incl/Excl	BILE	Cholesterol	MPG	DCP	pH	size	zp
1	N1	11	Incl	0	0.4	0.5	0.1	8.65	5.18	-54.29
2	N2	10	Incl	200	0.4	0.5	0.1	8.68	4.87	-65.08
3	N3	17	Incl	200	0.2	0.5	0.3	4.41	4	-36.74
4	N4	15	Incl	0	0.4	0.5	0.11	8.69	5.14	-59.89
5	N5	2	Incl	0	0.21	0.5	0.3	3.74	18.03	-44.27
6	N6	4	Incl	200	0.4	0.5	0.11	8.18	5.43	-61.8
7	N7	9	Incl	200	0.21	0.5	0.3	4.83	4.32	-42.93
8	N8	12	Incl	0	0.203333	0.5	0.3	3.89	12.68	-44.98
9	N9	6	Incl	0	0.333333	0.5	0.166667	8.09	4.98	-51.5
10	N10	7	Incl	0	0.266667	0.5	0.233333	8.03	5.12	-51.73
11	N11	13	Incl	66.6667	0.2	0.5	0.3	4.24	3.44	-41.29
12	N12	3	Incl	200	0.3025	0.5	0.2025	7.83	4.04	-51.06
13	N13	1	Incl	100	0.4	0.5	0.105	8.76	4.83	-61.14
14	N14	8	Incl	100	0.305	0.5	0.205	7.68	4.13	-46.04
15	N15	5	Incl	100	0.3025	0.5	0.2025	7.63	4.82	-51.22
16	N16	14	Incl	100	0.3025	0.5	0.2025	7.63	4.56	-49.91
17	N17	16	Incl	100	0.3025	0.5	0.2025	7.65	5.45	-50.28

B

Exp No	Exp Name	Run Order	Incl/Excl	BILE	Cholesterol	MPG	DCP	pH	size	zp	Peptide
1	N1	11	Incl	0	0.4	0.5	0.1	8.33	5.48	-57.85	61.8
2	N2	10	Incl	200	0.4	0.5	0.1	8.64	4.23	-60.29	41.6
3	N3	17	Incl	200	0.2	0.5	0.3	4.8	4.15	-38.04	73.1
4	N4	15	Incl	0	0.4	0.5	0.11	8.33	5.26	-63.35	52.6
5	N5	2	Incl	0	0.21	0.5	0.3	3.89	11.33	-41.36	36.9
6	N6	4	Incl	200	0.4	0.5	0.11	8.18	4.56	-60.04	47.6
7	N7	9	Incl	200	0.21	0.5	0.3	5.09	2.97	-36.75	36.5
8	N8	12	Incl	0	0.203333	0.5	0.3	3.78	11.21	-36.94	31.2
9	N9	6	Incl	0	0.333333	0.5	0.166667	7.57	5.49	-48.24	59.9
10	N10	7	Incl	0	0.266667	0.5	0.233333	7.83	4.91	-48.98	14.8
11	N11	13	Incl	66.6667	0.2	0.5	0.3	4.7	3.3	-35.38	45.7
12	N12	3	Incl	200	0.3025	0.5	0.2025	7.73	4.12	-50.09	22.3
13	N13	1	Incl	100	0.4	0.5	0.105	8.43	4.66	-63.75	36.1
14	N14	8	Incl	100	0.305	0.5	0.205	7.56	4.6	-47.62	72.5
15	N15	5	Incl	100	0.3025	0.5	0.2025	7.61	5.01	-49.19	61.6
16	N16	14	Incl	100	0.3025	0.5	0.2025	7.69	4.06	-45.78	60.8
17	N17	16	Incl	100	0.3025	0.5	0.2025	7.71	4.06	-50.5	86.2

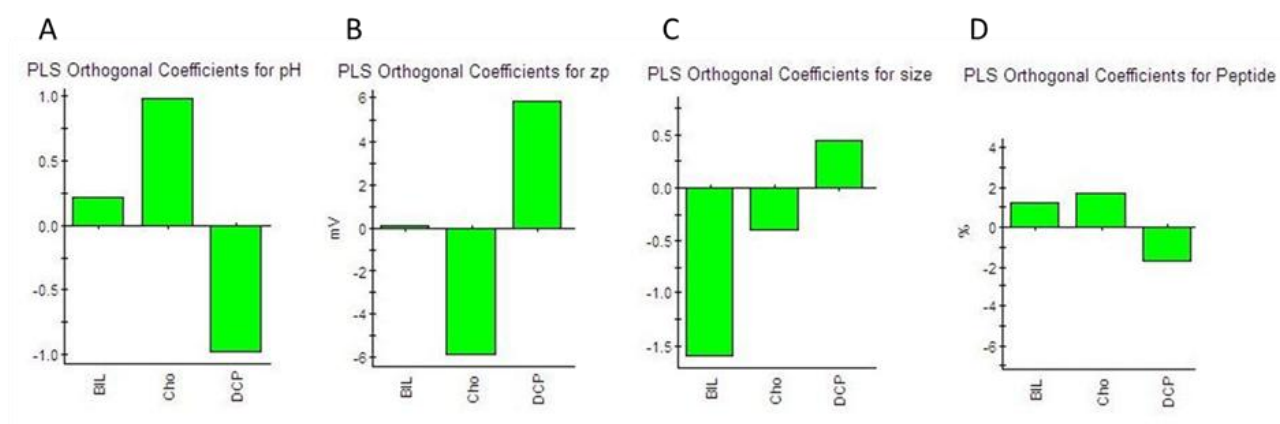


Figure 2.4: The partial least squares (PLS) orthogonal coefficients of the bilosome composition containing peptide which involved varying the different lipid ratios on **A)** pH, **B)** zeta potential, **C)** vesicle size and **D)** Peptide entrapment. The PLS plot takes into consideration several responses and fits a model showing the variation of the responses to the factors.

Table 2.2: ANOVA results for pH, zeta potential, vesicle size and peptide entrapment as a response (Y_1)

Response	<i>F</i> -value	<i>P</i> -value
pH	14.8585	0.001
Zeta	10.7774	0.002
Size	1.53747	0.292
Peptide	0.327196	0.939

Furthermore, in terms of vesicle size and peptide entrapment, statistically there is no significance of the lipids as shown in table 2.2. However, by taking the vesicle size coefficients from Figure 2.4C the bile salt has a negative impact that is to say that an increase in bile salt content in the formulation reduces the vesicle size of the bilosomes. Due to constraints placed on the DCP and cholesterol when designing the experiment, the total combined ratio of the two components does not exceed 5. In which case, when DCP content is used in its highest ratio the cholesterol is used in its lowest ratio with MPG kept constant at a ratio of 5. The individual effects of the formulation parameters will be looked into throughout the rest of this section.

2.3.1.1 The effect of formulation parameters on pH

Whilst it is recognised the pH can be modified after formulation, the effect on the formulation and hence the need for any pH modification was initially investigated, as the stability of formulations can be linked to their pH and formulations with and without the addition of the peptide antigen were considered.

Results from the ‘empty’ vesicles when directly compared to vesicles loaded with peptide antigen show very similar pHs (Table 2.1) suggesting that the addition of peptides made no notable effect on pH of the vesicle suspensions, as would be expected given the low volumes added to the formulations. However, the key factor shown to influence pH in both ‘empty’ and peptide loaded bilosomes was the concentration of DCP within the vesicles. Figure 2.5 shows that increasing the concentration of DCP within the bilosome formulation resulted in a decrease in pH, with formulations containing 1 molar DCP having pH values of over 8, whilst those containing high DCP values of 3 molar ratio having pH values of around 4 to 5 (Figure 2.5). The buffers used in the experiment were of pH 7.6 and pH 9.7, so the pH in theory should lie between 7.6 and 9.7. However this was not the case and figure 2.5 suggests that this was due to the DCP content of the bilosomes. These preliminary results suggest that increasing concentration of DCP may have resulted in the increased dissociation of the anionic surfactant which increased H^+ concentrations and therefore lead to a reduction in pH, However as both cholesterol and DCP concentrations were varied, this result was the starting platform for a series of further experiments where cholesterol content was kept constant and the DCP was varied (Section 2.3.2).

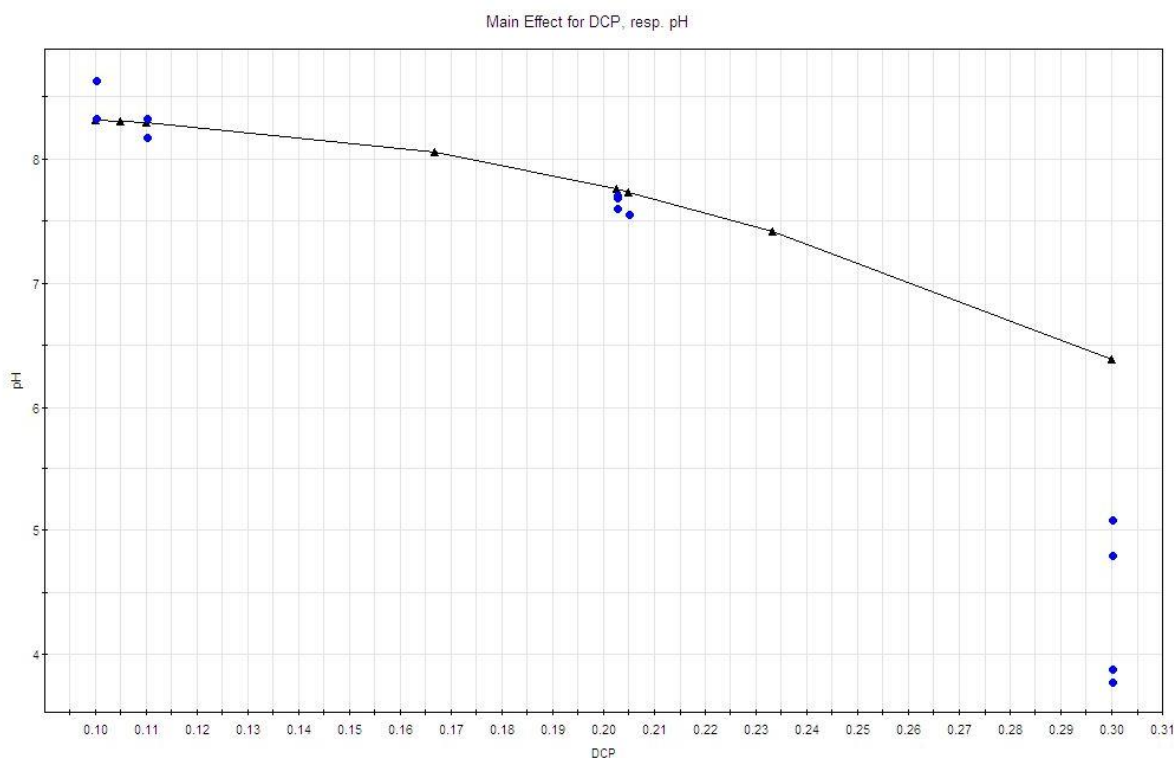


Figure 2.5: Main effects response for DCP upon the pH of the bilosomes. As DCP content is increased the cholesterol content decreases as the two lipid together must have an overall ratio totalling 5 (i.e. 0.5) Circles represent actual experimental values and triangles represent predicted values from the model.

2.3.1.2 The effect of formulation parameters on vesicle size

Given the importance of vesicle size in relation to both physico-chemical and biological attributes, it is important to elucidate the link between formulation and particle size such that it can be optimised. Vesicle size plays a crucial role in the final destination site of the vesicles, thus it was important to optimise vesicle size for the route of delivery. Vesicle size as a function of bile salt is presented in figure 2.6 and an initial observation suggests increasing bile salt reduces vesicle size. What this figure fails to indicate is that at each ratio of bile salt used, the lipid content varied. For example, by selecting 3 data points from Table 2.1 B, runs 11, 10 and 1 all have the same ratio of lipids (5:4:1 MPG:Chol:DCP respectively), however, the only thing which changed was the addition of bile salts. Run 11 contained no bile salt and resulted in a vesicle size of 5.45 μm and by increasing the bile salt concentration to 100 mM the vesicle size

reduced to 4.66 μm . A further increase in bile salt to 200 mM (run 10) resulted in a vesicle size of 4.23 μm .

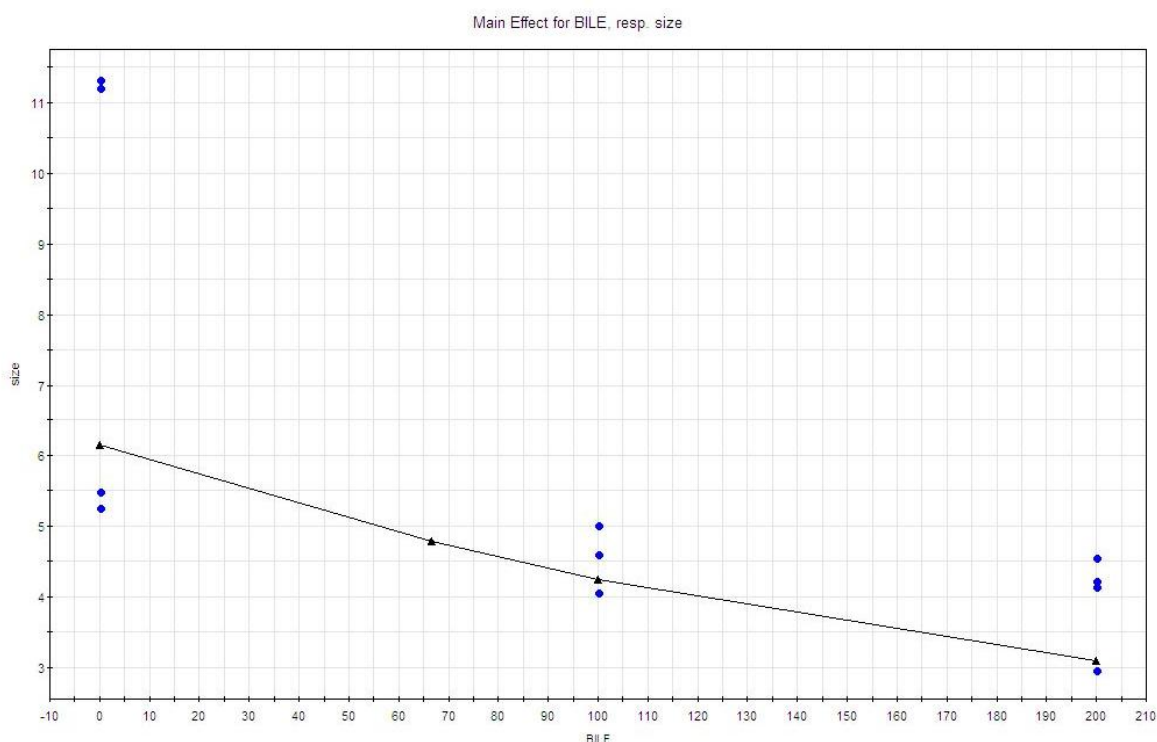


Figure 2.6: Main effect of the Bile salt upon vesicle size of formulations with different lipid ratios of MPG:Chol:DCP.

This same trend of bile salt influence for the large variation in vesicle size also applies when DCP is used in a ratio of 3 (Runs 2 and 9 from Table 2.1A). Runs 2 and 9 both have a lipid ratio content of 5:2.3 of MPG:Chol:DCP respectively with the only difference being that run 2 had 0 mM bile salt with a vesicle size of 18.03 μm and run 9 contained 200 mM bile salt which resulted in a vesicle size of 4.83 μm . These results from the DoE study once again gave another platform into the insight of how bile salt could be affecting the size of the vesicles and this study is also extended in the next section (Section 2.3.2).

Overall, from the formulations investigated it can be seen that the two components studied that make a notable impact on vesicle size is the concentration of DCP and bile salt, with the presence of peptide to the formulation having no impact at the

concentrations used. Previous studies by Chupin et al. (2002) on peptide interactions with bilayer vesicles showed that peptides bind to DCP increases the volume of the head group, thus lowering the charge (Chupin et al., 2002). To examine this further the effect of formulation on zeta potential was also investigated.

2.3.1.3 The effect of formulation parameters on peptide entrapment

Peptide loading within the vesicles is crucial as the higher the entrapment, the more peptide can be present at the target site, which in turn, can result in using lower doses overall. As a result, to investigate the effect of formulation on peptide loading, the formulations outlined in Table 2.1B were also tested for peptide association using a modified ninhydrin assay. The bilosomes were centrifuged using a protocol developed in section 2.2.4 and assayed using the ninhydrin assay (Section 2.2.6) to determine peptide entrapment. The ninhydrin assay was used to measure entrapment of peptides and proteins within bilosomes; this assay eliminated any interference of lipids or surfactants when compared to other assays, such as the BCA assay and Lowry method (Brewer et al., 1995, Lowry et al., 1951). Results from the study showed that no individual component was significant in influencing peptide entrapment within the vesicles (Figure 2.4D). The peptide entrapment was not dependent upon any of the excipients used to form vesicles and was proven to occur independent of lipids used. As a result, the vesicle size and peptide entrapment was investigated to determine if the larger vesicles result in increased peptide association as presented in figure 2.7.

The results in figure 2.7 showed no correlation between peptide entrapment and vesicle size, suggesting that antigen loading may be determined by other factors. The lipid ratio and bile salt concentration changes for each formulation in table 2.1B and the resultant peptide entrapment differs. An example of this is the triplicate control experiment from

Table 2.1B which showed peptide entrapment at 60 %, 61 % and 86 % for the same ratio of lipids used.

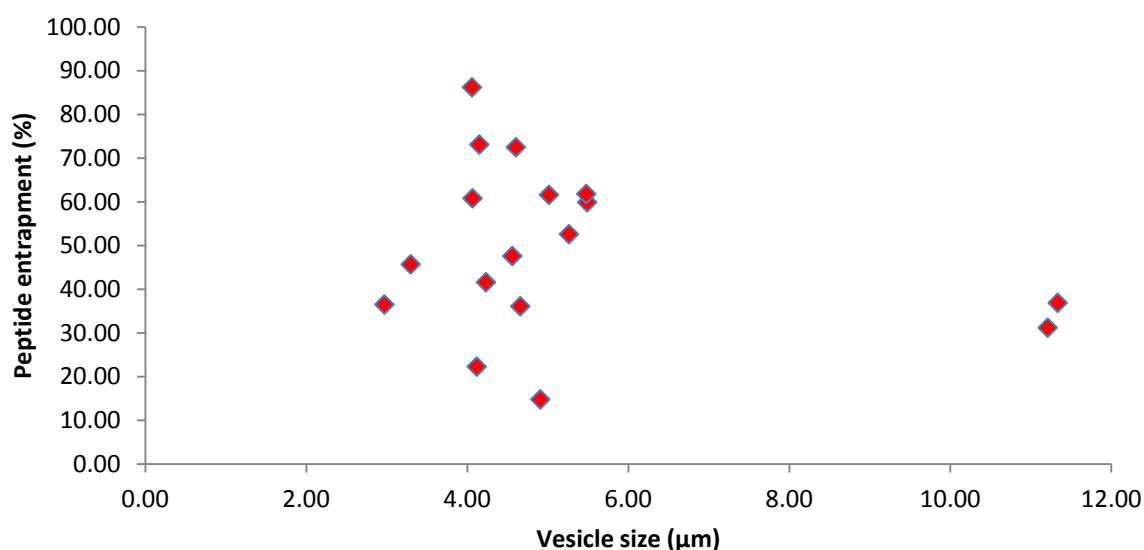


Figure 2.7: The effects of vesicle size upon the peptide entrapment within the vesicles.

Studies by Brgles et al, (2008) also confirm findings that anionic liposomes did not change in vesicle size upon increasing the amount of entrapped protein; however, they found that for cationic liposomes entrapment efficiency is highly dependent on ionic strengths of buffers used (Brgles et al., 2008). Furthermore, Brgles et al, (2008) report that negatively charged OVA in anionic liposomes is not affected by ionic strength of buffers used and that the entrapment is presumably due to hydrophobic interactions between the OVA and phospholipid bilayers as the vesicle size of the liposomes remains unchanged. One of the underlying reasons for variation in entrapment efficiency could be due to the change in lipid ratios used. For example, studies have shown that the increase in cholesterol content within MLVs can result in increased content of citocoline (Puglisi et al., 1992). Furthermore the effect of varying lipid content within this design of experiment study can result in variation in number of bilayers within the vesicles which in turn affects the volume of the aqueous core and the bilayer volume hence affecting loading efficiency.

2.3.1.4 The effect of formulation parameters on zeta potential

Zeta potential is the nearest experimental approximation of surface potential, as both the surface potential and stern potential are difficult to measure. Thus the zeta potential is the potential at the surface of shear which is measured by electrophoresis and is a good indicator of stability of dispersed systems. This charge at the vesicle surface affects the distribution of ions within the region which causes an electric double layer to be formed. When an electric current is passed through each particle and its ions in the region move throughout the solution. The rate and extent of movement within the stern layers and the surrounding medium determine the zeta potential. Therefore, zeta potential is a function of the vesicle surface charge which is calculated by the adsorbed layers at the interface and the nature of the surrounding medium. Figure 2.8 shows that a highly negative zeta potential in the vesicle formulations tested, can be achieved with lower ratios of DCP to cholesterol (e.g. as in the case of run 2 in Table 2.1A), which appears counter intuitive, as it would be predicted that increasing DCP and reducing cholesterol content would increase the anionic nature of the vesicles. This trend in zeta potential values was apparent for both the peptide loaded and 'empty' vesicles with no significant difference in zeta potential between the two comparable sets.

Figure 2.4 A,B showed the orthogonal coefficient plots for pH and zeta potential and the most significant factors were clearly the DCP and cholesterol which are graphically presented in figure 2.8. More detail of the effect of the two components is shown in figure 2.9 which shows response surface and contour plots of DCP content against cholesterol content and the effects on pH and zeta potential. Figure 2.9 illustrates that the interaction of the two components DCP and cholesterol has an impact upon the pH where it ranges from a low of 3.5 to a high of 8.5 and zeta potential which has a range of -38 to -64 mV depending upon the lipid content within the vesicle.

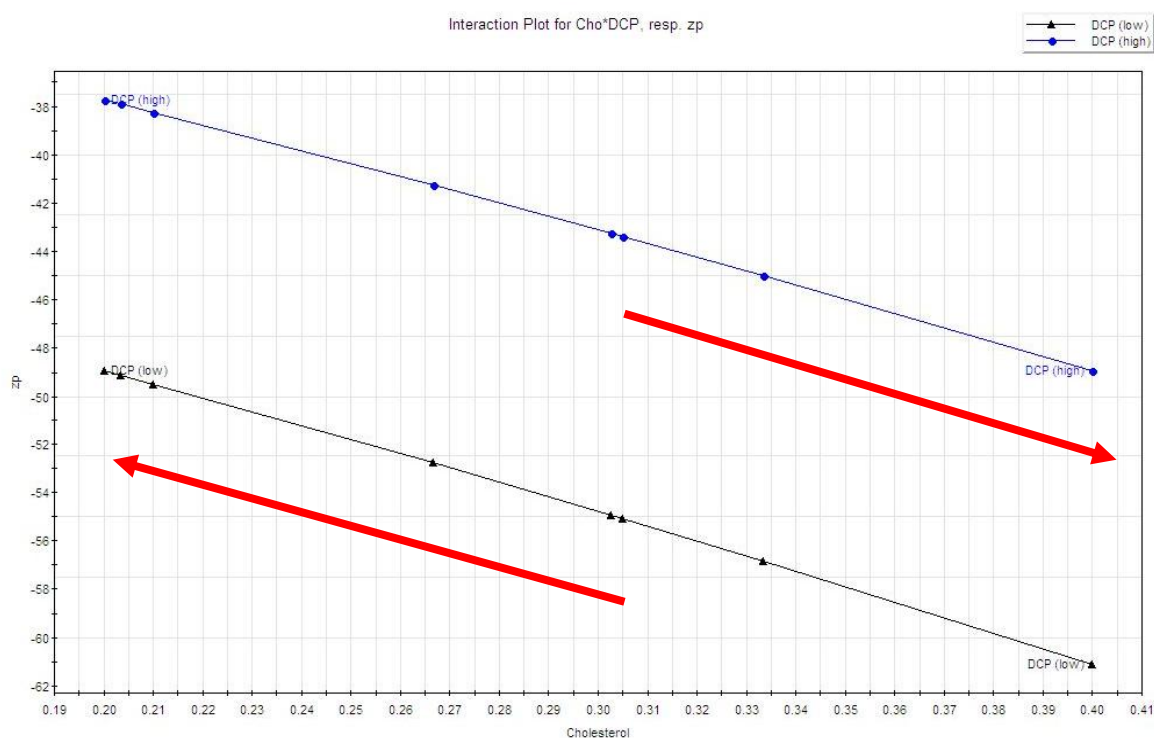


Figure 2.8: The effect of DCP content against cholesterol content on the zeta potential of the bilosome vesicles. Low DCP = 0.1 ratio and high DCP = 0.3 ratio. NB. Chol and DCP together should add to 0.5 therefore the red lines show predictions of the design.

The pH can be closely linked with the zeta potential in terms of DCP and cholesterol as figure 2.9B shows that when DCP is used in its highest ratio with cholesterol at its lowest the pH becomes more weakly acidic with a pH of 5.01 and below. When compared to the zeta potential (Figure 2.9D) the same ratios of lipids results in a less negative zeta potential occurring with a reading of -40 mV. As the two lipid contents change, the relationship of pH and zeta potential are linked with a general trend resulting in the more alkaline the pH the more negative the zeta potential. Studies by Junyaprasert et al, (2008) show that DCP inclusion to salicylic acid at a pH of 5 showed greatest stability with the highest zeta potential compared to niosome vesicles prepared at pH 3 which had reduced surface charge (Junyaprasert et al., 2008). Zeta potential can help reveal the degree of repulsion between vesicles of a similar charge. In addition, the presence of DCP resulted in a charged interface thus causing electrostatic repulsion between adjacent bilayers, resulting in an increased distance between the bilayers

hence, causing rise of the internal aqueous compartment of MLVs (Kulkarni et al., 1995). The presence of a positive or negative zeta potential implies that the suspension is more likely to be stable and may also prevent aggregation of vesicles due to repulsive forces between the vesicles (Freitas and Müller, 1998, González-Rodríguez and Rabasco, 2011).

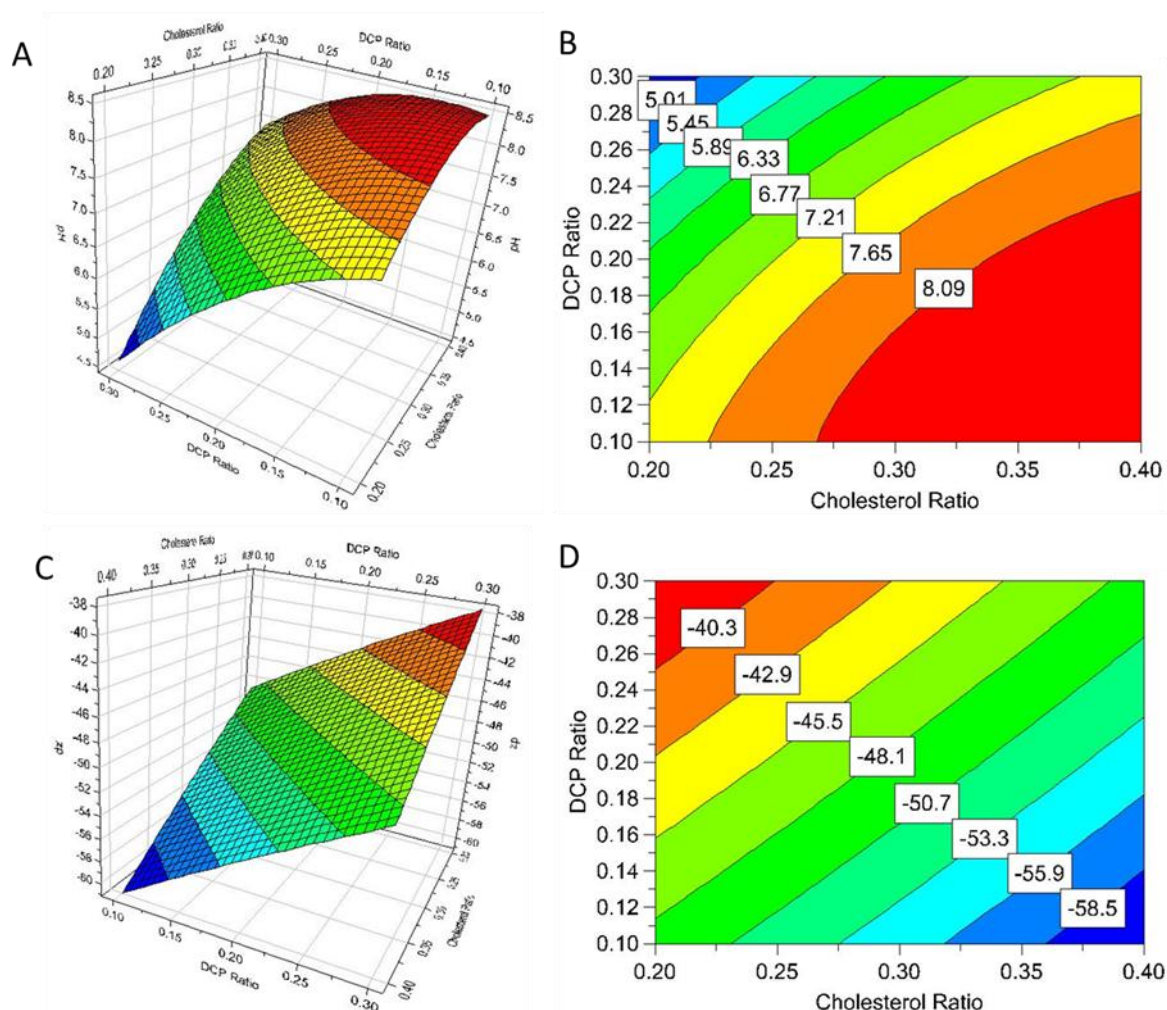


Figure 2.9: Response surface plots for cholesterol and DCP content against pH and zeta potential. **A)** Response surface plot showing the effect of DCP and cholesterol content on the pH, **B)** Contour plot of DCP and cholesterol effects on the pH, **C)** Response surface plot showing the effect of DCP and cholesterol content on the zeta potential, and **D)** Contour plot of DCP and cholesterol effects on the zeta Potential. All response plots are taken where bile salt content is at 100mM as bile salt content does not influence pH or zeta potential. Due to restrictions on software all ratios add up to 1 (ie. A ratio of 4 for cholesterol would appear as 0.4 on the diagrams, MPG is kept constant on the design at a ratio of 5 which is 0.5)

2.3.1.5 Optimisation of bilosomes for oral vaccine delivery

The use of DoE allows a database to be generated which allows the use of high throughput screening of specific parameters that are being investigated. For oral vaccine delivery, an optimum vesicle size range of 4-10 μm is required for vesicles to retain within the target site of the Peyer's Patches (Eldridge et al., 1990), a highly negative zeta potential (Norris et al., 1998), and as high an antigen loading as possible. As a result these optimum response parameters were programmed into the DoE database and as a result the surfactant combination which best fits these responses were given as a 5:4:1 MPG:Chol:DCP respectively with 70-120 mM bile salt for maximum coverage to meet all criteria (Figure 2.10). Bile salt levels above 120 – 200 mM result in a reduction in coverage to meet criteria (figure 2.10). The red sections indicate the optimum concentrations where all criteria are met and the increase in bile salt results in less criteria being met hence the reduction in red zone and increased coverage within other zones.

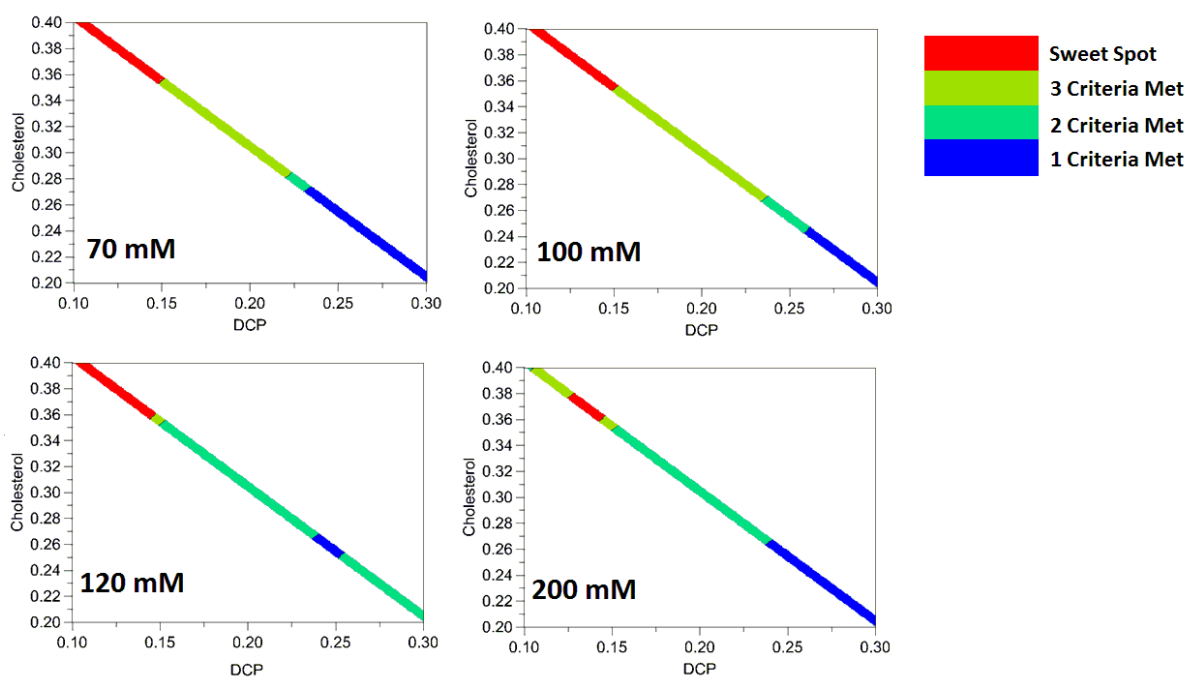


Figure 2.10: Sweet spot plot representing optimal DCP and Cholesterol ratio with 70-200 mM bile salt. (Parameters set, Size- 4-6 μm , pH 7-9, zeta potential -55 - -65mV, Peptide entrapment 40-60%).

To further examine the trends observed in section 2.3.1, an in depth study was undertaken where MPG and cholesterol content were kept constant at a ratio of 5:4 respectively with 100 mM Bile salt based on the optimisation using sweet spot plot (Figure 2.10) with the DCP content changing from a molar ratio of 1 to 3. This study was used to indicate to what extent the DCP lipid influences the pH and zeta potential of the bilosome formulations and validate the DoE model.

2.3.2 Validation of DoE responses

So far, the results have shown that from the design of experiments study in section 2.3.1 where three excipients (cholesterol, DCP and bile salt) are varied there are specific trends that can be linked to certain excipients. It is crucial to isolate these trends to individual excipients hence the rationale for the next study was to maintain three constant parameters and change the fourth. An example of this included keeping MPG:Chol at a ratio of 5:4 with bile salt concentration constant at 100 mM and then to change the ratio of DCP from 1-3 in increments. The aim of the study was to determine whether the trends in the DoE study (Section 2.3.1) can be repeated but by changing only one variable. Once again the parameters looked at were pH, vesicle size and zeta potential.

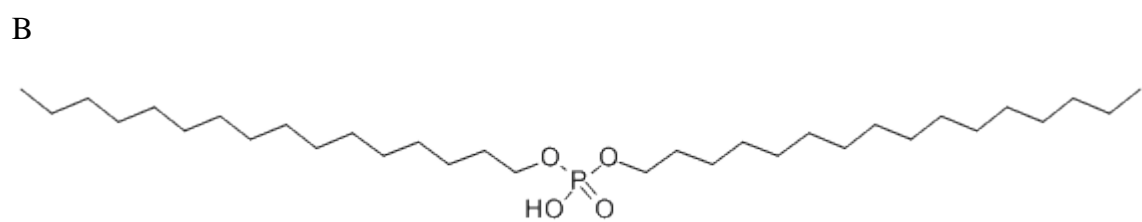
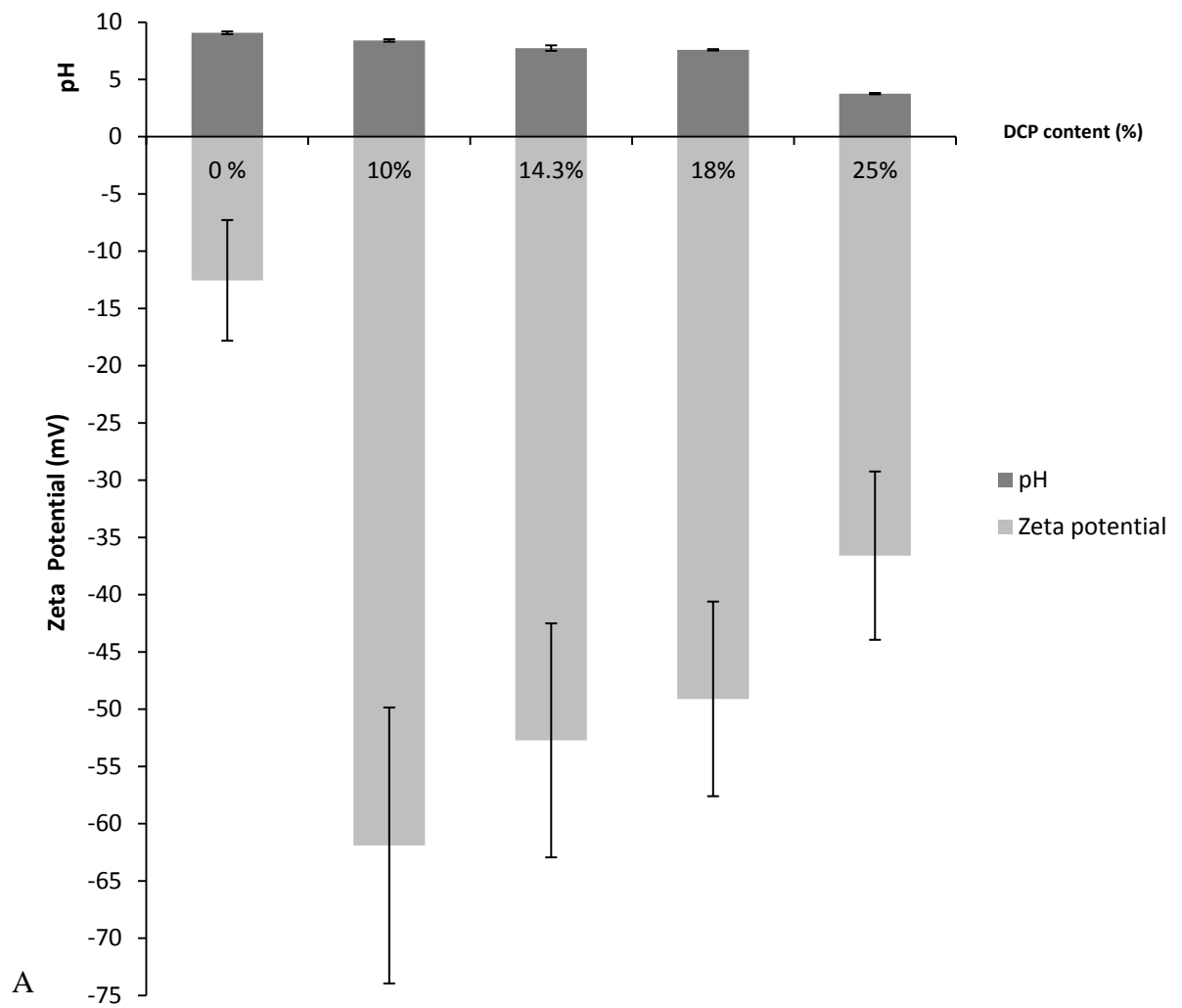
2.3.2.1 The effect of DCP content within bilosomes on pH and zeta potential

From the design of experiments study, an in depth study regarding the effect of DCP as an individual lipid on the pH, zeta potential and vesicle size was carried out. Within this study the MPG and cholesterol ratios were kept constant at a ratio of 5:4 respectively with the bile salt content also being constant at 100 mM. Results in figure 2.11 show that pH decreases and the zeta potential becomes less negative when there is an increase in DCP content (from 10 to 25 mol%) which is comparative to the design of

experiments study where cholesterol and bile salt were changed (Figure 2.9). A potential cause of this could be due to the degree of ionisation of DCP, an amphiphilic molecule, when incorporated in to the bilosomes. DCP (Figure 2.11B) has an OH group which is present in the non-dissociated form, but when in solution the H^+ ion can be released. The removal of this H^+ ion gives the DCP its negative charge, and hence anionic zeta potential, when in solution and in turn the H^+ ion gets taken up by the sodium bicarbonate buffer which is present in the formulation. As the DCP ratio is increased within the formulations, there are more H^+ ions within the formulation which are being taken up by the buffer, however, there is a buffering capacity. The sodium bicarbonate (Na_2CO_3) buffer when split up becomes $2Na^+ + CO_3^{2-}$, hence for every mole of buffer there would need to be 2 moles of DCP. As a result, when the DCP is in a ratio of 25 % there becomes a 2:1 ratio of DCP: H^+ ions in relation to the buffer. Hence, this is the point at which buffer capacity is lost and there becomes a reduction in pH due to excess H^+ ions and no buffer to take them up. Between 10-18 % DCP ratio the pH remains constant (Figure 2.11A), However as the DCP concentration is increased the pH drops due to the excess H^+ ions now in the formulation. The DCP molecule has a pK_a of ≤ 2.6 (Uchegbu, 2000) thus, ionisation of the phosphate group takes place at $pH \leq 2.6$ resulting in the large change in surface charge as represented in figure 2.11C. This acidic environment could inhibit further dissociation of DCP, therefore limiting its ability to impart an anionic charge into the vesicles. This can explain why with increasing DCP content, a reduction in the anionic zeta potential was noted rather than an increase in negative charge as would be expected (Figure 2.11).

The impact of DCP on a vesicles suspension's pH were also shown by Manosroi et al, (2003) when developing a liposome formulation for tranexamic acid (TA) with various lipid compositions including negatively charged DCP lipid and a positively charged

stearylamine lipid (Manosroi et al., 2003). One of the features of their study showed that negatively charged lipids (including DCP) gave low pH values ranging from 3.4-7.1 compared to the positively charged lipids (pH 7.6-7.9). The addition of TA to the negatively charged lipids results in a rise in pH due to the neutralisation of the DCP hydronium ion from the hydroxyl ion released from the ionisation of the TA. The negative charge resulting from DCP also resulted in smaller liposomes due to the neutralisation of charges with the TA hence producing smaller vesicles with a 10 % entrapped TA To confirm the direct link between the pH of the bilosome suspension and the measured zeta potential, bilosomes of negative zeta potential and high pH were subjected to acid titrations and the effects of pH on zeta potential investigated (Figure 2.11C).





C

Figure 2.11: **A)** Effect of changing DCP content on the pH and zeta potential of the bilosomes. **B)** Structure of the anionic DCP lipid and **C)** A pH titration graph of 5:4:1 ratio of MPG:Chol:DCP respectively with 100 mM bile salt containing peptides showing the effect of pH upon the zeta potential of the bilosomes n=3.

Figure 2.11C involved making a batch of bilosomes with a 5:4:1 ratio of MPG:Chol:DCP respectively with 100 mM bile salt and peptides with a starting pH of approximately 9.4. This study was carried out on a Malvern zetasizer ZS and a MPT-2 Auto titrator. Three titrant vials were added to the instrument which were 1 M HCl, 0.1 M HCl and a 1 M NaOH solution where the software was set up and designed to lower the pH of the bilosome formulation by 0.5 units at a time and then the zeta potential to be measured. So far in previous studies we have shown that an increase in DCP content results in a more acidic pH. By carrying out this study, bilosomes were exposed to specific pH levels and the zeta potential measured (Figure 2.11C). The results show that as the pH is raised from 1 to 10, the zeta potential of the vesicles changes from approximately -15 mV to -120 mV confirming the controlling role of pH on the anionic nature of the bilosomes (Figure 2.11C). Therefore by varying the DCP content in the bilosome formulations, this modulates the pH of the suspension system which in turn

impacts on the zeta potential of the bilosomes, thereby confirming that a continued increase in anionic surfactant content may not always promote increasing anionic zeta potential of vesicles.

In terms of how this impacts on biological responses, the zeta potential has been shown to influence the uptake of bilosomes in studies by Tomizawa et al, (1993) who have shown that negatively charged vesicles are preferably taken up by the Peyer's patches (Tomizawa et al., 1993). Therefore, by incorporating appropriate levels of DCP into the formulation, it gives the bilosomes their negative charge, which can be retained throughout the various pH ranges of the GIT (Figure 2.11C) suggesting that this will aid in the uptake of the bilosomes by the Peyer's patches at the range of pH seen across the GIT.

To summarise, the intentions of the study were to confirm the previous studies and the design of experiments work (Section 2.3.1) and to determine how well design of experiments can be applied for the current formulations. This study of DCP content on pH confirms some of the early findings of the study (Section 2.3.1) by showing a similar trend to the design of experiments study, however in terms of absolute values there is no overlay of the results. This shows that design of experiments by factorial design is useful in showing trends of specific lipids to the system but it was not absolute in this study. The change in pH is closely linked with the zeta potential of the vesicles. In terms of vesicle size, which remains unchanged as DCP content is increased suggests that the addition of DCP does not alter the vesicle size (data not shown) except when no DCP is used. This concept of pH and zeta potential are important factors in the delivery of bilosomes as they are delivered orally and are subjected to extreme pH environments along the GIT. It is crucial that at this stage that the bilosomes are stable and intact at low pH's most importantly with the antigen being protected and stable. The aims of

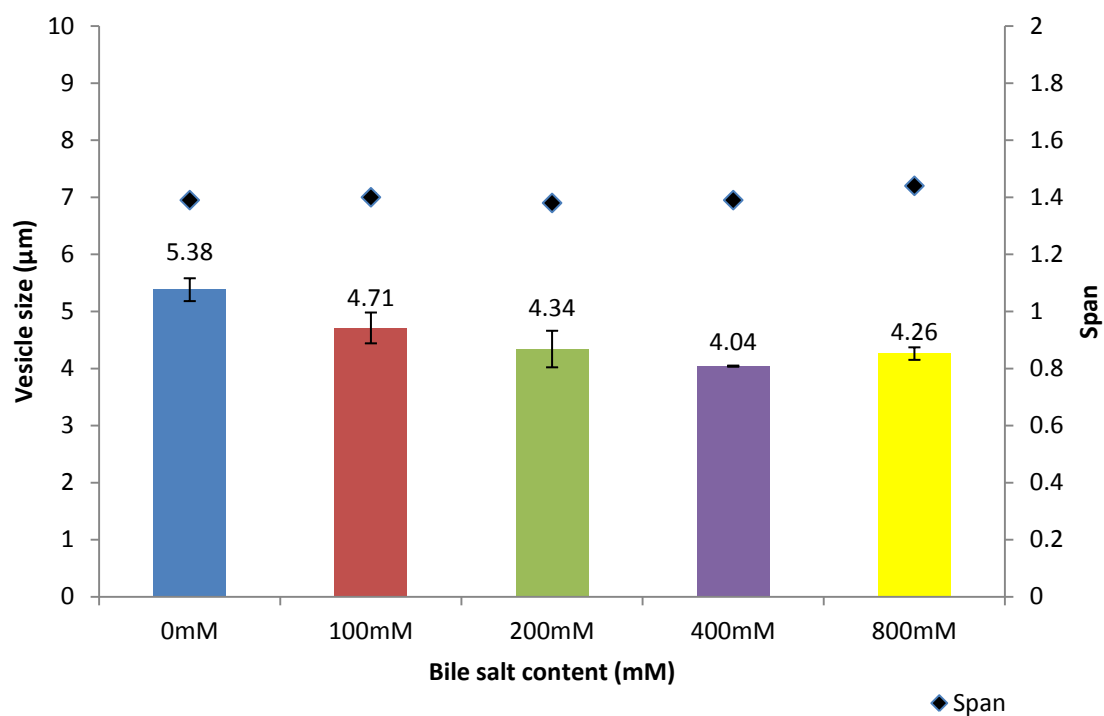
non-ionic surfactant vesicles are to protect the antigen under extreme pH conditions and from the intestinal bile to allow vesicle and antigen to remain intact upon entry at the target site.

2.3.2.2 Effect of bile salt concentration on the vesicle size

The vesicle size range is crucial as it determines the end location of the vesicle within the gastro intestinal tract (GIT). The target for bilosomes is the immuno-competent cells within the gut, which are known as Peyer's patches. Studies by Eldridge et al, (1990) on vesicle size uptake in the Peyer's patches show that uptake is dependent on the vesicle size and hydrophobicity. The results from their experiments indicate that a vesicle size of 10 μm or less is taken up by the Peyer's patches, however particles with a size less than 4 μm can traffic to other tissue cells by a process of lymphoid drainage (Eldridge et al., 1990). As a result, particles larger than 4 μm reside within the Peyer's patches. Therefore, the target size for bilosome formulations designed for oral vaccination would be approximately 4-6 μm so that they can reside within the Peyer's patches and to exert their therapeutic effect.

Given that the design of experiments study previously demonstrated (Section 2.3.1) that the most important factor controlling vesicle size is the bile salt, a new set of formulations were prepared where all formulations in this study were made with a 5:4:1 ratio of lipids (MPG: cholesterol: DCP) but with the bile salt concentration changing. The bile salt concentrations used were 0 mM, 100 mM, 200 mM, 400 mM and 800 mM. It is to be noted that working with the 800 mM bile salt was difficult as the bile salt solution became very viscous.

A



B

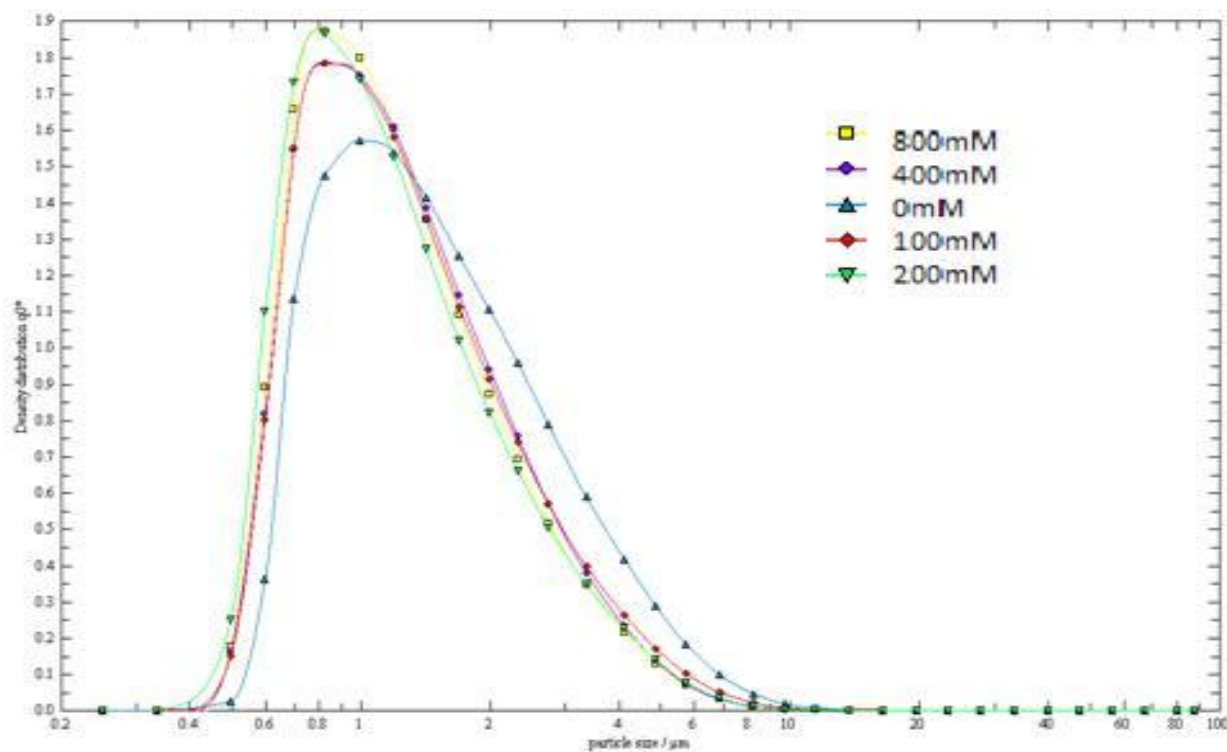


Figure 2.12: Niosome vesicles containing bile salts showing the **A)** Effect of bile salt content on mean vesicle size distribution (VMD) with span and **B)** Number frequency of the formulations with increasing bile salt content in relation to the VMD (n=3).

Figure 2.12A shows that as the bile salt concentration is increased the vesicle size decreases. A previous study incorporating bile salt within lipid vesicles by Chen et al, 2009 have also shown the same trend of decreasing vesicle size upon increased bile salt concentration; this was attributed to enhanced flexibility and a lowering of surface tension between the vesicles when bile salts were present (Chen et al., 2009). An increase in the flexibility of the lipid bilayer causes a softening effect to occur, which impacts upon the stability of the vesicles such as multilamellar vesicles (MLV). The vesicles in the study are in microns which fit into the MLV category and with the increase in fluidity the MLV are susceptible to form small vesicles upon an external force.

This notion of smaller vesicles forming from MLVs due to the increased fluidity, hence causing instability of the MLV, can be visualised in figure 2.12B which shows the number distribution of the vesicles upon increasing bile salt concentration. Figure 2.12B shows the number frequency of the vesicles at the specific vesicle sizes within the formulation. As the bile salt content is increased, the number of smaller vesicles also increases, suggesting that as we lose the bigger vesicles we are gaining an increase in smaller vesicles (Figure 2.12B).

The niosome formulation (0 mM bile salt) shows a density distribution towards a larger vesicle population as represented by the number frequency of these vesicles in relation to the formulations containing bile salt. By examining the 200 mM bile salt formulation there is a shift of the density distribution curve towards the smaller vesicle size range. In addition to the shift, the peak is higher suggesting that there are more vesicles of that size range within the formulation. The formulation containing the highest bile salt concentration of 800 mM shows the highest peak, further suggesting that there are more numbers of smaller vesicles within the formulation. The addition of bile salts to the

5:4:1 formulation shows a decrease in mean vesicle size as shown in figure 2.12A, however also shows an increase in the smaller vesicles obtained as shown in figure 2.12B.

In general, there needs to be an optimal concentration of bile salt to be incorporated within the vesicles without influencing vesicle uptake within the Peyer's patches. This present study shows that across the bile salt concentration range tested, the formulated bilosomes were generally in the 4-6 μm particle size range therefore should reside within the Peyer's patches as required for oral vaccine delivery.

2.3.3 Vaccine efficacy challenge study using ferret model

After optimising the 5:4:1 (MPG:Chol:DCP respectively) with 100 mM bile salt formulation for oral vaccine delivery, bilosomes containing the synthetic peptides directed against influenza viruses were administered to ferrets orally on days 0, 3, 14, and 17, and 14 days and later challenged with a clinical rHA isolate of influenza (Figure 2.13). To investigate the efficacy of the vaccine and comply with 3R protocols, the key correlate of protection, the median temperature differential was measured to follow protection against fever and inflammatory cell counts in nasal washes (Figure 2.14 and 2.15).



Figure 2.13: Dose regime for oral vaccination of a ferret challenge study. (Studies were carried out at Variation Biotechnologies, Canada).

The bilosome vaccine incorporating the synthetic peptides shows a reduced median temperature differential change compared to a dose of empty bilosomes and the commercial vaccine administered intramuscularly (IM) suggesting that the antigen containing bilosomes via the oral route show strong results in providing effective protection from fever (Figure 2.14).

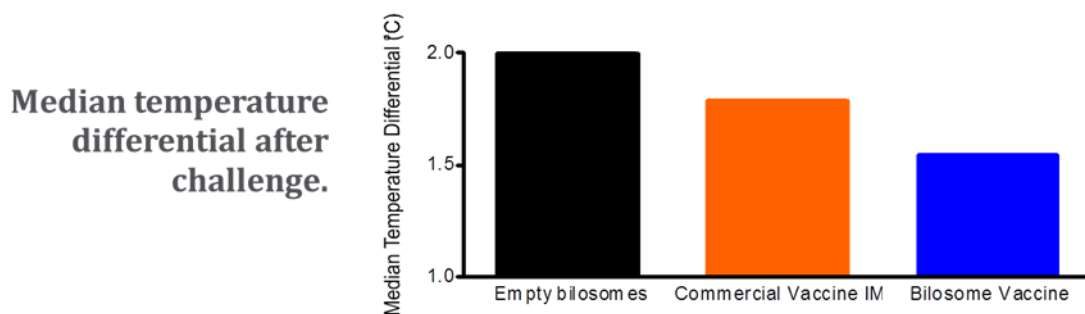


Figure 2.14: Median temperature differential change after challenge using a clinical isolate of Influenza A. (Studies were carried out at Variation Biotechnologies, Canada).

After analysing nasal washes for inflammatory cells, the bilosome formulation containing the synthetic peptide vaccine compared to empty bilosomes resulted in a

reduction in viral cell load counts compared to bilosomes administered without antigen (Figure 2.15). Moreover, the bilosome vaccine was comparable to the commercial vaccine administered IM. Thus the bilosome formulation containing the influenza vaccine has protected against fever and suppressed lung inflammation to extents comparable to the commercial vaccine (IM) showing promising results for vaccine delivery via the oral route.

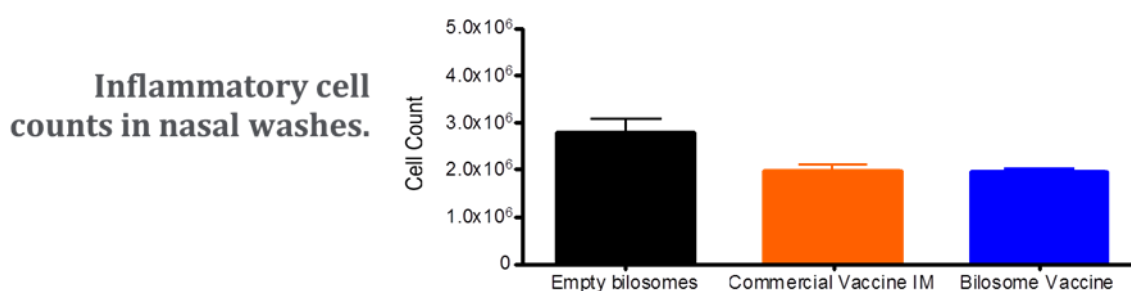


Figure 2.15: Inflammatory cell counts after nasal washes of the three formulation groups. (Studies were carried out at Variation Biotechnologies, Canada).

Research has shown that, when delivering antigen alone via the oral route, this is not sufficiently immunogenic and thus requires the use of adjuvants and carriers to enhance immunogenicity (Azizi et al., 2010). In addition, the reduced immunogenicity can be accounted by the lack of control of the antigen reaching intestinal surfaces which can be due to milieu of the GIT and the poor uptake by the epithelial cells (Devriendt et al., 2012). The oral biodistribution of an influenza antigen to be further investigated within the GIT will compare free antigen dose with formulating the antigen with the bilosome vesicles which has the potential of allowing increased antigen at the target site by offering uptake via specialised M cells within the Peyer's patches. Previously, Amorij et al, (2007) have shown that the intragastric delivery of recombinant H3N2 antigen alone induced low HI titres, significantly lower than the antigen delivered IM (Amorij et al., 2007). The use of bilosomes as vaccine carriers with antigen, has shown effective mucosal immunity, compared to the delivery of free antigen, where a study by Shastri et

al, (2012) have also shown that formulating antigen into microspheres offers higher mucosal antibody production compared to free antigen delivered via the oral route (Shastri et al., 2012). The use of a potent adjuvant or delivery system is critical in obtaining a sufficient immune response which is comparable to a corresponding response IM (Amorij et al., 2007). Once again, the use of a delivery system offers a more targeted approach of delivery and renders antigens more immunogenic as they offer antigen protection against degradation and have a longer residence time within the GIT.

2.4 Conclusions

An optimisation study by the use of design of experiments has been successful in suggesting possible lipid interactions upon specific responses measured. This lead to further in depth studies on individual lipids and from the results it can be concluded that the pH and zeta potential are linked together and are controlled by the anionic DCP lipid which is used in bilosomes to give the net negative charge to allow uptake and vesicle formulation. In terms of vesicle size which remains unchanged as DCP content is increased, suggests that the addition of DCP does not alter the vesicle size. However, the bile salt contributes to the decrease in vesicle size and increase in smaller vesicles which can be due to increased fluidity within the bilayer causing slight instability hence the formation of smaller vesicles. DoE is a useful tool to understand trends and give an insight into process parameters; however, the absolute values predicted by the software out of the experimental range were not consistent with those of formulations prepared.

Thus, the optimised formulation predicted by the software was validated and reproduced on several occasions showing reproducibility of vesicle size, pH, zeta potential and antigen loading. The 5:4:1 of MPG:Chol:DCP respectively containing 100

mM bile salt was subsequently formulated (assuming full incorporation of all the excipients) with the synthetic peptides for an oral challenge study in the ferret animal model. The results of the study were comparable to the commercial vaccine administered IM thus showing a promising platform into the delivery of vaccine antigens via the oral route to induce mucosal immunity.

Chapter 3

Development of low temperature emulsification methods for the manufacture of non-ionic surfactant based vesicles.

Publications related to this Chapter:

1) Wilkhu, J., A. Vangala, Mohammed, A., Perrie, Y. (2013). Designing Nonionic Surfactant Vesicles for the Delivery of Antigens for Systemic and Alternative Delivery Routes. Immunomic Discovery of Adjuvants and Candidate Subunit Vaccines. D. R. Flower and Y. Perrie, Springer New York. **5**: 205-232.

3.1 Introduction

3.1.1 Preparation of non-ionic based vesicles

Non-ionic vesicles can be prepared through a range of methods, including the lipid hydration method (Azmin et al., 1985, Baillie et al., 1985), the reverse evaporation technique (Guinedi et al., 2005), the dehydration-rehydration method (Brewer and Alexander, 1992) and the molten-lipid emulsification method (Mann et al., 2004). Whilst the last method offers the advantages in terms of easy of scale up, any thermal induction methods to support the emulsification of lipids presents the risk of enhanced degradation promoted by thermal energy hence, it is vital to understand analytically the thermal properties of the surfactants used in addition to characteristics such as vesicle size and charge.

To further investigate the manufacture of non-ionic based vesicles via the molten-emulsion method, the surfactants used to form niosomes were at set ratios which were optimised in chapter 2. Previous studies on niosome vesicles, which also employed a ratio of 5:4:1 of MPG:Chol:DCP respectively to produce vesicles, have suggested that this mixture can be melted at 120 °C or 140 °C which is below the melting point of cholesterol (150 °C) (Bennett et al., 2009, Mann et al., 2004). To understand the thermal events occurring during the process, there are a range of techniques available.

3.1.2 Differential Scanning Calorimetry for thermal Analysis

Differential Scanning Calorimetry (DSC) has been widely used in its application in understanding the thermal characteristics of materials (Bouzidi et al., 2005) where an insight into a range of thermal properties including phase transitions including glass transition (T_g) and heat capacity changes are presented. The output of data is measured as an endothermic or exothermic transition as a function of temperature. An example a

typical thermogram is shown in Figure 3.1 where the MPG surfactant was used on a controlled heat cool heat cycle.

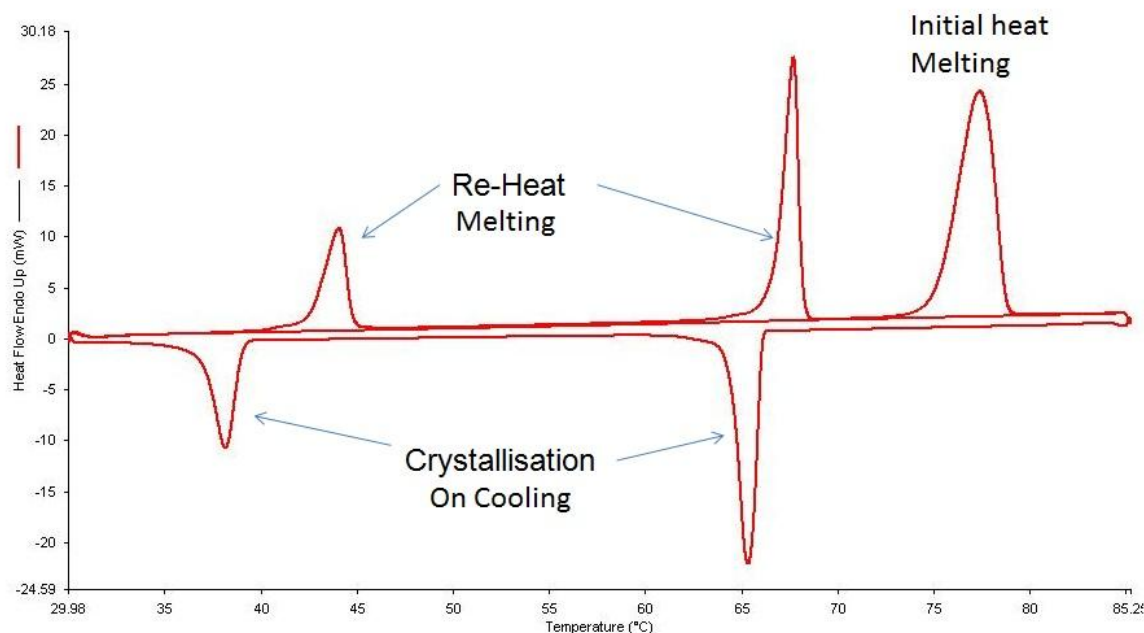


Figure 3.1: A typical DSC thermogram of a powdered surfactant. MPG surfactant showing melting upon heating and then crystallisation upon cooling. Isotherm shows Endothermic going up hence melting peaks are up (Scan rate = 10 °C/min).

DSC works on the principle of measuring the difference in heat energy of a sample pan against a reference pan under the same experimental method and atmospheric conditions (Demetzos, 2008). Hence when analysing powders the reference pan is empty and when analysing liquid vesicle formulations the reference pan should be of the same aqueous buffer the vesicles are formed from. This is vital as any thermodynamic events that take place due to the buffer, the sample pan will also detect the same changes hence will not interfere with the heat enthalpy.

As a result, phase transitions which are key factors in the production of a range of drug delivery systems including bilayer vesicles can be determined by DSC which provides an insight into the gel to liquid phase transition of bilayer vesicles. The application of

thermal energy to lipid bilayer vesicles such as liposomes and niosomes results in a change in state which is known as the gel crystalline – gel liquid phase transition. This phase transition is represented Figure 3.2 which shows the major transitions that take place when heating bilayer vesicles.

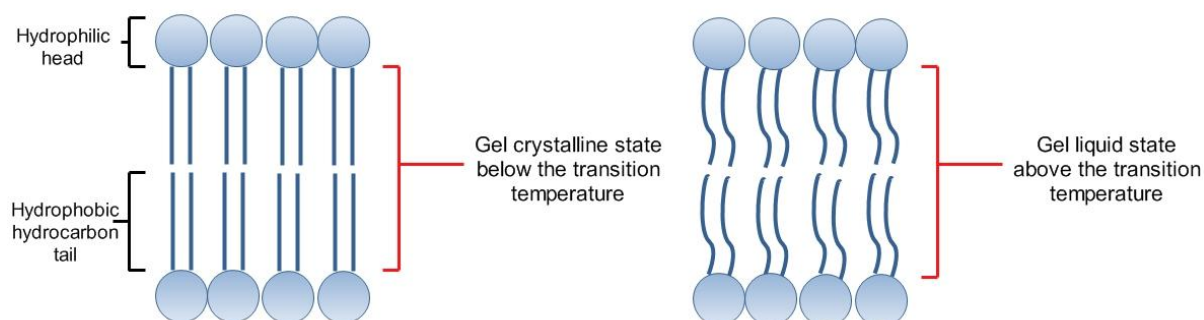


Figure 3.2: Schematic representation of phospholipid arrangement above and below the transition temperature.

The formation of liposomes results in the production of rigid bilayered vesicles below the transition temperature of the lipid(s) used. The rigidity of the vesicles is attributed to the properties of the hydrocarbon tails of the phospholipids which crystallise below the main phase transition temperature (Figure 3.2). Upon heating to the phase transition temperature, these crystalline tails effectively “melt” and become fluidic tails thus giving rise to the gel liquid state. The main phase transition of bilayer vesicles depends upon the phospholipids used and the addition of any other components to the vesicles such as cholesterol, which is known to remove the main phase transition temperature and change bilayer fluidity (Oldfield and Chapman, 1972). Addition of cholesterol has shown to increase the retention of fluorescent dyes hence resulting in increased stability of vesicles and preventing leakage of entrapped material from the vesicles (Kirby and Gregoriadis, 1980). As a result, by knowing the phase transition temperature of bilayer vesicles by the use of DSC techniques, this helps in the manufacture of vesicles as they

need to be formed above the transition temperature. Furthermore, the properties of bilayer vesicles can be controlled and manipulated to achieve the desired effect.

3.1.3 Aims and Objectives

In this chapter, the aim was to investigate the temperatures required in the process of forming vesicles using the melt method as first outlined by Conacher et al, (Conacher et al., 2001). The objectives were to determine:

- Whether the melting of non-ionic surfactant vesicles (NISV) can take place prior to homogenisation at lower temperatures preventing degradation of surfactants.
- Whether DSC, TGA and Langmuir models can provide a rationale to determine whether this is possible and then niosome vesicles will be prepared by melting the surfactants at different temperatures prior to homogenisation.
- The formulations will be analysed for physico-chemical instabilities over a period of time after storage of the formulations at 4 °C, 25 °C and 40 °C. Empty niosomes and niosomes formulated with OVA were also tested for a period of 28 days.

3.2 Materials and Methods

3.2.1 Surfactants

A range of non-ionic surfactant based bilayer vesicles were prepared by high shear homogenisation and analysed using several materials and methods. The surfactants used in the study were Monopalmitoyl glycerol (MPG) (Larodan Labs, Sweden), Synthecol (Chol) (Sigma Aldrich, UK) and Dicetyl Phosphate (DCP) (Sigma Aldrich, UK). The

aqueous buffer sodium bicarbonate (Sigma Aldrich, UK) was buffered to pH 7.6 using a 1 M sodium hydroxide solution (Sigma Aldrich, UK).

3.2.2 TGA of surfactant blends at the two melting temperatures

Thermogravimetric analysis (TGA) was also employed to understand thermal degradation and lipid/surfactant decomposition when elevating the temperature of the samples (Skala et al., 1997). The surfactants were assessed prior to formulation of the vesicles as the results would provide an indication of an initial melting temperature to be used to form the vesicles with.

A 5:4:1 ratio of surfactants (MPG:Chol:DCP respectively) were weighed and placed into a glass vial and mixed. A sample of this mixture was placed onto the Perkin Elmer TGA and analysed to 95 °C, 120 °C or 140 °C and held isothermally for 10 minutes at each of the three temperatures respectively. 120 °C and 140 °C were chosen to replicate currently published melting temperatures whilst 95 °C was chosen to determine the effects at a lower melting temperature. All samples were repeated in triplicate to determine degradation and reproducibility, and all formulations were carried out using nitrogen gas and air.

3.2.3 DSC of individual surfactants and the surfactant blend

The surfactants were analysed individually in the solid state using a TA Instruments Q200 Thermal Analysis DSC. The DSC was calibrated using sapphire and indium for a cell constant and temperature calibration based on the heat flow and type of cooler in place on the system. The individual surfactants were weighed using an analytical balance into T-Zero aluminium pans and then hermetically sealed ensuring the weight between the triplicate samples was kept constant to ensure accurate enthalpy data. All

experimental runs were started at an initial temperature of 20 °C purged under nitrogen gas with a scan rate of 10 °C /minute to 120 °C.

After the individual surfactants were analysed for melting, different blends of the surfactant mixtures were further analysed based on a design of experiments template created where the ratio of surfactants was changed. The surfactants were weighed in the correct ratios into a glass vial and mixed. A sample of this blend was placed into the pan to see the overall melting temperature of the surfactant blend together. The method used for DSC had a heating rate of 10 °C/min over a temperature range from 0-160 °C with the purge gas being nitrogen.

3.2.4 Preparation of non-ionic surfactant based vesicles

To prepare the bilayer vesicles the surfactants in the powder form (307.5 mg) were mixed at the appropriate ratio (5:4:1 of MPG:Chol:DCP respectively), melted in an oil bath at 120 °C for 10 minutes, and while maintaining the molten lipid mix, vesicles were created by the addition of 5.45 mL of 25 mM sodium bicarbonate (Sigma Aldrich, UK) buffer pH 7.6 (60 °C) and homogenised for 5 minutes using a Silverson machines homogeniser model #L4RT. After 5 minutes a pre-incubated (60 °C) solution of OVA (0.55 mL) was added to the vesicles and further homogenised for 5 minutes. Upon cooling, the niosome formulation was incubated for 2 hours in an incubating mini-shaker (VWR) with gentle shaking at 220 rpm.

3.2.5 Preparation of monolayers using Langmuir trough system

Langmuir monolayer studies have been widely used to understand the packaging of surfactants and lipids when mixed together by spreading the insoluble amphiphilic molecules in chloroform onto an aqueous water subphase (Gopal and Lee, 2006). The purpose of the Langmuir is to generate surface pressure isotherms to determine whether

the surfactants will form uniform monolayers and relate these findings to bilayers when formed as niosome vesicles.

Monolayer studies of the individual surfactants (MPG, Chol and DCP) and a mixture of surfactants in the ratio 5:4:1 of MPG:Chol:DCP respectively were carried out using a KSV mini trough Langmuir system (KSV Instruments Ltd, Helsinki, Finland) equipped with a platinum Wilhelmy plate in an isolated area. Ultrapure water 18 Ω (Milipore, UK) formed the subphase used within these studies and the temperature of the trough was kept constant at 20 ± 1 °C using an external water circulation system. Stock solutions of the individual surfactants were prepared at a 0.5 mg/mL in chloroform and a mixture was also prepared in chloroform at the set ratio. The method used was adapted from Ali et al, (2010) where 20 μ L of the surfactant stock solutions was spread onto the air/water interface using a glass Hamilton syringe precise to ± 0.2 μ L (Ali et al., 2010). Upon spreading of the samples onto the interface the chloroform was left to evaporate and the hydrophilic barriers were set to close at a speed of 10 mm/min to form monolayer isotherms. Each sample was run once until it reached its collapse pressure and then triplicates of the sample were taken by thoroughly cleaning the trough with chloroform and then restarting the experiment. The data was analysed on the KSV instruments software.

3.2.6 Determination of vesicle size and zeta potential

The vesicle size distribution was determined using laser diffraction on a sympatec 2005 (Helos/BF) cuvette analyser. 20 μ L of the bilosome suspension was diluted into the cuvette with 40 mL double distilled water. The zeta potential, which is an indirect measurement of the vesicle surface charge, was measured in 1.5 mL double distilled

water at 25 °C on a Zeta Plus Brookhaven Instrument. 15 µL of the niosome suspension was mixed in 1.5 mL double distilled water and then analysed.

3.2.7 Determination of OVA entrapment

The OVA loading was determined in the vesicles after separation via centrifugation, produced by the addition of 300 µL of the niosome sample in a 3.9 mL Beckman centrifuge tube filled with double distilled water for a period of 40 minutes at 354,000 X g (TLN-100 rotor), using the Ninhydrin assay at a wavelength of 560 nM. Following centrifugation, supernatant was removed with a pipette and pelleted OVA loaded vesicles were re-suspended and 100 µL of the sample used for the assay is placed into a 1.5 mL polypropylene micro-centrifuge tube (in triplicate). The tubes were then dried at 110 °C on a digital heat block (VWR) and when dry, hydrolysed by 150 µL of 13.5 M NaOH (Sigma Aldrich, UK) to each tube.

The tubes were then heated at 110 °C for 20 minutes and then cooled to room temperature. 250 µL glacial acetic acid (Fisher Scientific, UK) was added to the samples to neutralise the NaOH and 500 µL ninhydrin (Sigma Aldrich, UK) solution was added to each tube, vortexed and then placed on the digital heat block for 20 minutes at 110 °C. 250 µL aliquots of the samples from these tubes were transferred to fresh micro-centrifuge tubes containing 750 µL of propan-2-ol: water (Fisher Scientific, UK) (1:1 in v/v) and vigorously vortexed. The 1 mL sample was transferred into a semi-micro cuvette and the absorbance was read and recorded at a wavelength of 560 nM on a Jenway 6405 UV/Vis spectrophotometer. The amount of peptide present (µg/mL) is determined from a standard curve which is also made at the same time as carrying out the ninhydrin assay of the samples.

3.2.8 Appearance and morphology

Images of the formulations in their glass vials were taken on a digital camera to visualise the appearance of the formulation at the time intervals. A Zeiss Axiovert A1 upright light microscope using a 40x objective lens is used within this study to capture images to represent the morphological changes to the vesicles over a time period. 15 μ L of the sample is placed onto a glass slide with a cover slip on top and is then analysed using Axiovision software 36A. Scale bars within the article represent 10 μ m and a 40x objective is used throughout the stability study.

3.2.9 Stability study

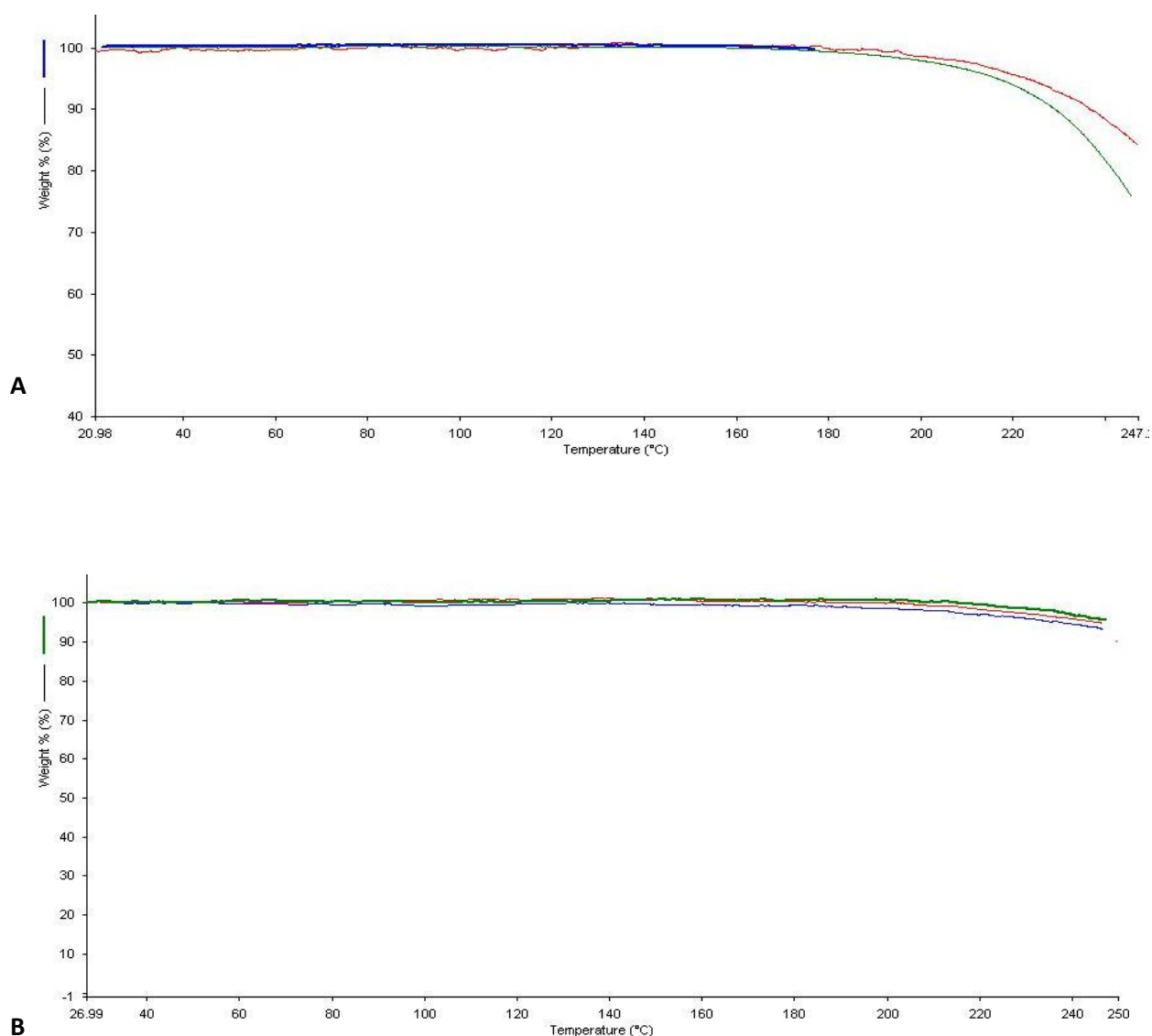
Batches of niosomes were prepared by method stated in section 3.2.4 either by melting the surfactants in an oil bath set at 95-98 °C or 120-125 °C. Upon formation of vesicles the formulation was split into three glass vials with 2 mL of formulation in each. 6 formulation batches were prepared from fresh for the OVA loaded vesicles and 3 formulation batches were prepared for the control. Each formulation was split and placed into 4 °C, 25 °C and 40 °C incubators and were analysed for vesicle size after a 10 second vortex, zeta potential and pH at time points for a period of 28 days.

3.3 Results and discussion

3.3.1 TGA of components and mixture

TGA is an analytical technique that is performed on the surfactants to determine changes in weight in relation to change in temperature. TGA depends on three main measurements which include weight, temperature and the temperature change. TGA studies for the components was conducted to determine whether there was any decomposition or weight loss at the temperatures employed in the manufacture of the vesicles (Skala et al., 1997). It is essential that the components do not degrade at the

temperatures of use, as this could lead to impurities within the formulation or by-products which could be toxic as the end goal is to test the formulations *in vivo*. Therefore, it is necessary to establish that all of the components used are stable and can withstand the temperatures used during production methods and these are studied in figure 3.3.



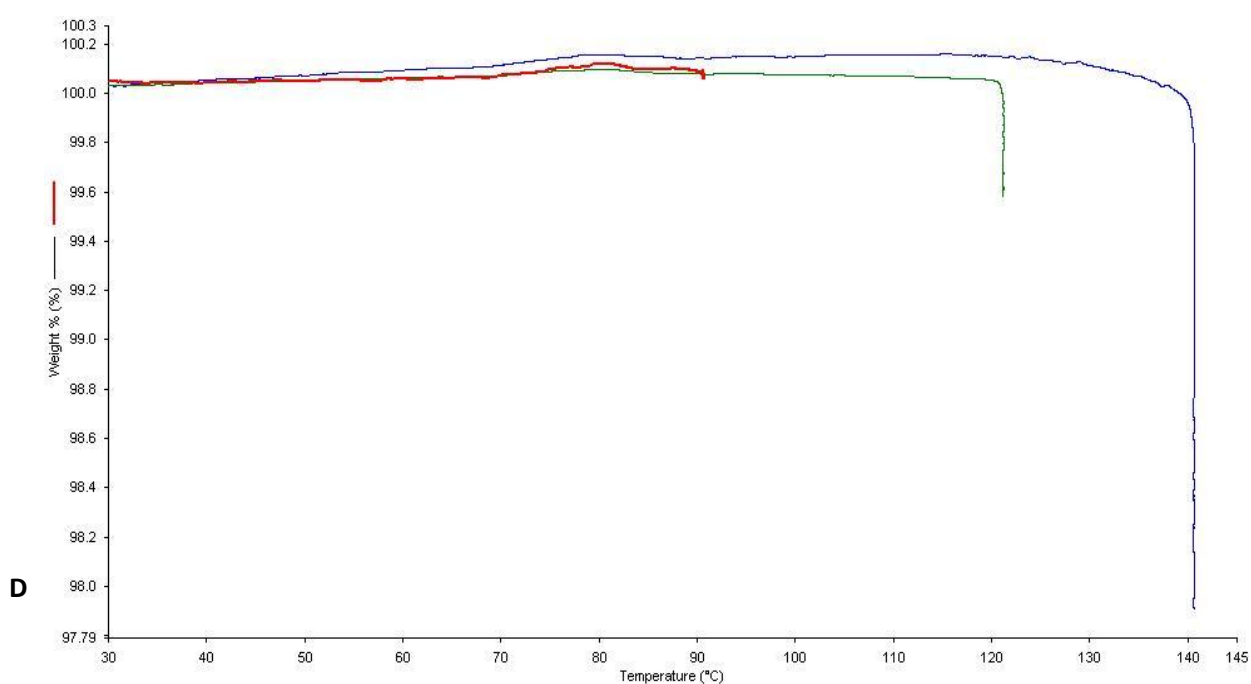
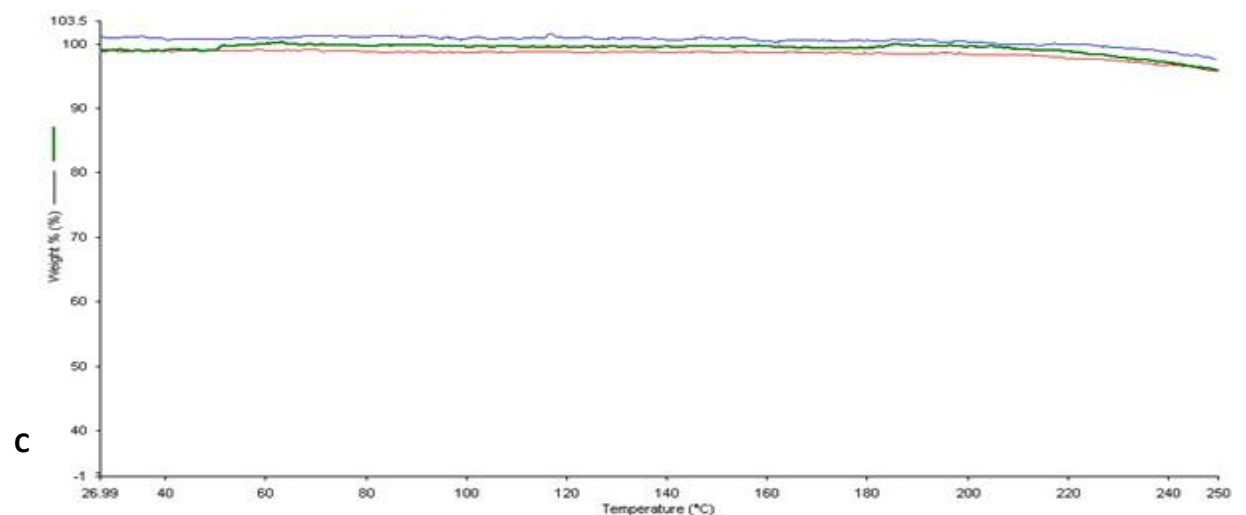


Figure 3.3: TGA Isotherms of the individual surfactants from 30-250 °C showing % degradation. **A)** MPG, **B)** Cholesterol, **C)** DCP and **D)** 5:4:1 (MPG:Chol:DCP) mixture where red line ramp to 90 °C and hold for 10 min, green line ramp to 120 °C and hold for 10 min and blue line ramp to 140 °C and hold for 10 min (Scan rate = 10 °C/min) n=3.

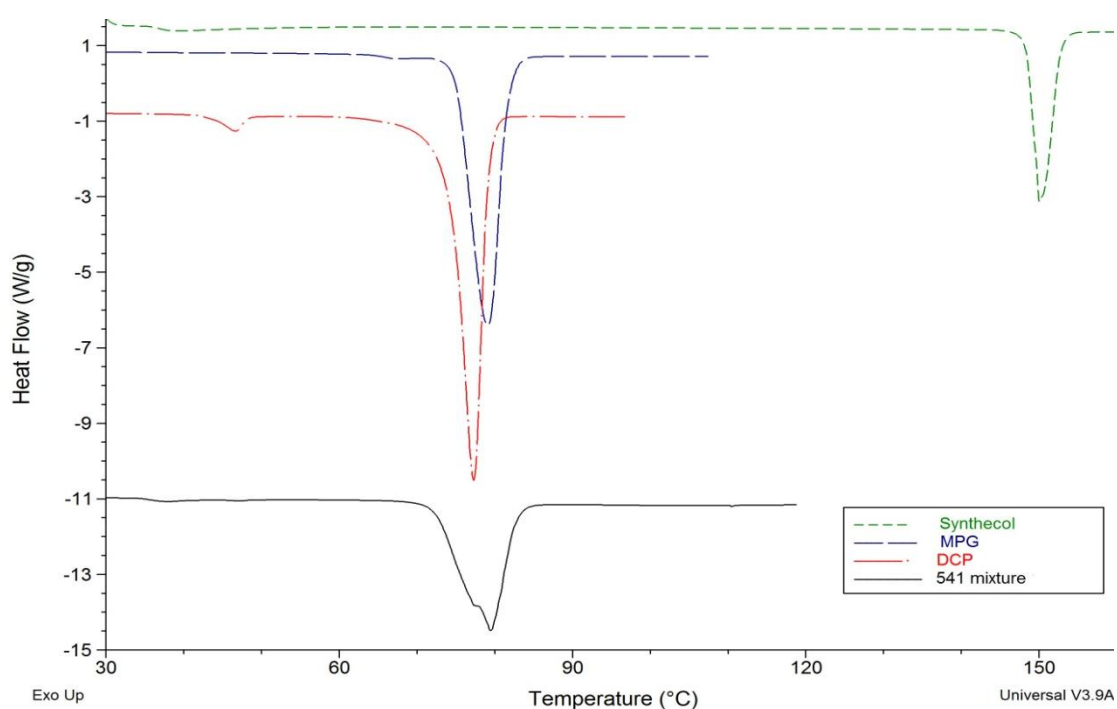
Figure 3.3 A-C show the replicate TGA isotherms of the individual surfactants MPG, cholesterol and DCP respectively. The heating range was taken from 30 °C to a final temperature of 250 °C which exceeds the working temperature range used to produce niosomes. The results show that the individual components show no degradation up to temperatures of approximately 180 °C (Figure 3.3 A to C), which is above the

temperatures used in the manufacturing method (120 °C) outlined in Section 3.2.4. However, when combining the surfactants into a 5:4:1 mixture of MPG:Chol:DCP respectively (Figure 3.3D), the surfactant mixture shows degradation at temperatures lower than the individual components with changes in weight being measured at 120 °C (Figure 3.3D). Previous studies have melted the surfactant mixtures at temperatures of 120 °C and 140 °C (Mann et al., 2004, Mann et al., 2009). However, Figure 3.3D suggests that this may have an impact on the surfactants integrity if they are held at this temperature for extended periods, as the TGA isotherms show that when heating the mixture to 140 °C and holding for 10 minutes there is a resultant weight loss of the mixture of 2.3 % (Figure 3.3D). Furthermore, when heating to 120 °C and holding isothermally for 10 minutes a weight loss of 0.6 % is observed. Studies by Othman et al, (2011) on the thermal effects of addition of corn starch to polyvinyl alcohol (PVOH) blends also demonstrates that different ratios of the mixtures significantly impact the thermal properties (Othman et al., 2011). TGA analysis showed that at 385 °C the pure PVOH showed maximum weight loss, and upon addition of corn starch to the PVOH this temperature significantly decreased to 374 °C and upon further substitution to 225 °C, thus showing that composite mixtures suppress the thermal stability of the initial PVOH due to the molecular interactions between the compounds (Othman et al., 2011). It is unclear whether this loss of the mixture plays an important role in the formulation of vesicles; however, this can be avoided by melting the component mixture between 95-98 °C which shows a mixture weight loss of 0.01 % after holding isothermally for 10 minutes at 95 °C (Figure 3.3D).

3.3.2 DSC of components and mixture

To further investigate the thermal characteristics of these mixtures, they were also investigated by DSC to determine the melting properties and to identify what temperatures could be employed to melt the lipid mixture without degrading the components. Figure 3.4 A, represents the thermograms of the individual components in their solid state, and a mixture blend of a 5:4:1 ratio of MPG:Chol:DCP respectively, also in the solid state. From Figure 3.4, the DCP component has a melting onset temperature of 74.23 °C with a T_{max} of 77.32 °C, followed by the MPG component with a melting onset of 74.92 °C with a T_{max} of 79.06 °C. The cholesterol component shows a melting onset greater than the components MPG and DCP of 148.74 °C with a T_{max} at 150.05 °C. Based on these component thermodynamic properties, it would suggest that to achieve a molten mixture of a blend they would need to be melted at a temperature in excess of 150 °C to allow for the cholesterol to melt.

A



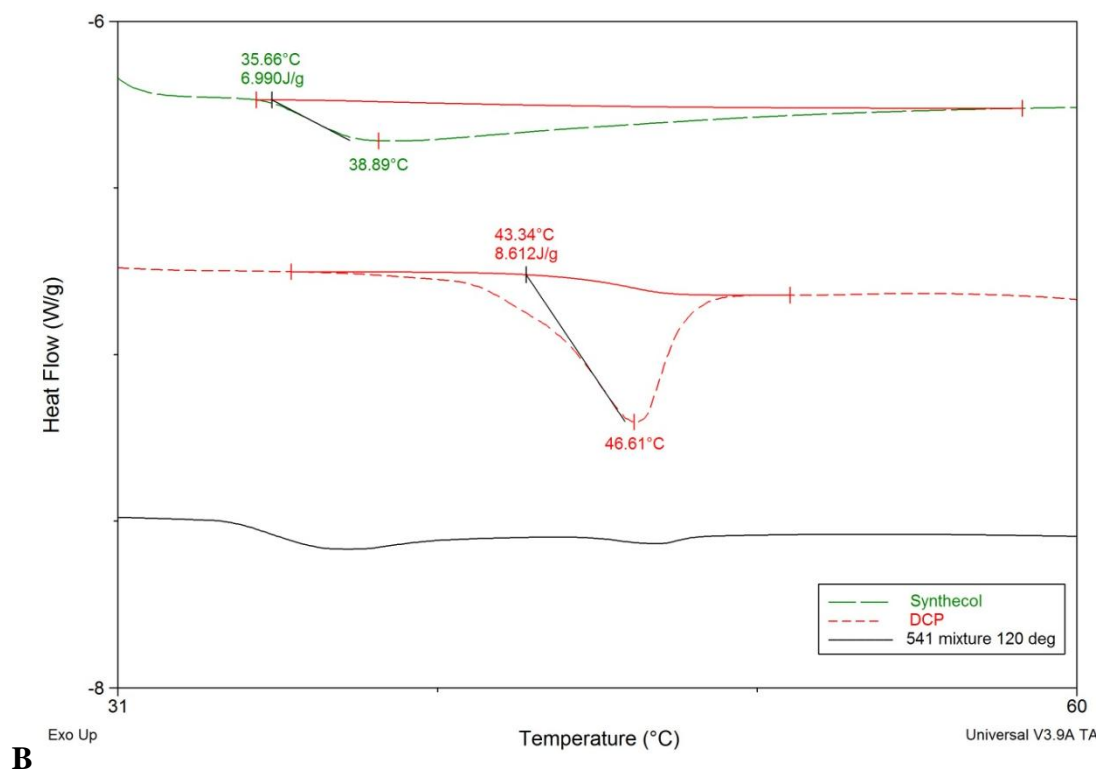


Figure 3.4: DSC thermogram of the surfactants and the corresponding mixture. **A)** Individual surfactants showing melting upon heating and a mixture of the 5:4:1 (MPG:Chol:DCP) powder blend. **B)** Thermogram of cholesterol, DCP and the 5:4:1 (MPG:Chol:DCP) mixture between 30-60 °C showing pre transitions. Isotherm shows exothermic going up hence melting peaks are down (Scan rate = 10 °C/min samples run in triplicate).

The results (Figure 3.4A) show that the cholesterol component has the highest melting point of 150 °C, whilst MPG and DCP melt at 79 °C and 77 °C respectively. However, when these three lipids were mixed at a 5:4:1 weight ratio (MPG:Chol:DCP), the mixture has a melting onset temperature of 72.26 °C with a complete melt (T_{max}) at 79.40 °C (Figure 3.4A). An initial study was carried out where the surfactant mixture was taken to 160 °C and no thermal events occurred after 100 °C suggesting that complete melting of the mixture has occurred below this temperature. Figure 3.4B represents the thermogram for DCP, Synthecol and the mixture of 5:4:1 weight ratio MPG:Chol:DCP respectively and reproducible pre transitions/ slow sub-gel transition within the components are visible which are much smaller than the main transition and in cases cannot be characterised in molecular terms (Bilstonen and Lichtenberg, 1993).

When analysing the samples under hot stage microscopy at these pre transitions, movement of the components occur and slight changes in the structure are noted (Figure 3.7).

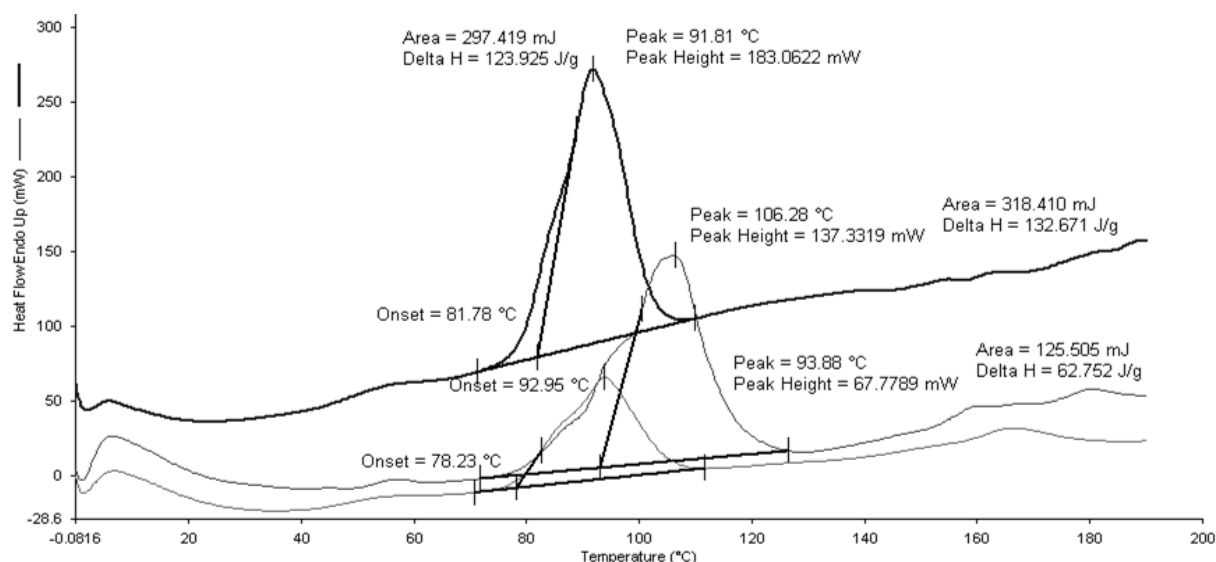


Figure 3.5: Hyper DSC thermogram of the mixture of 5:4:1 (MPG:Chol:DCP) n=3.

To determine whether cholesterol melted or solubilised within the solid state lipid mixture upon heating prior to formulation into vesicles, hyper DSC was used on the surfactant mixture MPG:Chol:DCP (5:4:1 respectively) to try to separate the thermodynamic events from any kinetic events, such as dissolving at a scan rate of 500 °C/min. The results from the hyper DSC give rise to a large broad melting peak which contains multiple peaks representing the surfactant mixture with no presence of a melting peak at the cholesterol melting point temperature (149 °C), indicating that the cholesterol integration has taken place prior to its melt (Figure 3.5). Faster heating rates are employed to provide insufficient time for dissolution/ solubilisation to take place, therefore should represent clear endotherm peaks at the melting points of the individual components (Gramaglia et al., 2005). This result would indicate that the scan rate of 500 °C/min was not fast enough as the rate of dissolution of cholesterol took place at a much faster rate than 500 °C/min. The use hot stage microscopy (section 3.3.3) further

looks into the melting of the surfactant blend where cholesterol is in contact and not in contact with the surfactant blend and the subsequent effect of temperature on melting.

To understand this reduction in melting temperature observed in figure 3.4A, ratios of synthecol and DCP were further studied using DOE whilst MPG was kept constant. A design of experiments study was carried out using a D-optimal design using the DoE software (MODDE), and the ratios of the worksheet are presented in Table 3.1. No constraints were placed on this design which allowed the cholesterol to be used in any ratio with the DCP hence effects of the individual components could be analysed for melting onset temperature, melting enthalpy, crystallisation enthalpy and reheat enthalpy. MPG was always kept constant at a ratio of 5.

Results (Table 3.1) show that when the ratio of cholesterol was increased relative to the MPG and DCP, the onset of overall melting point decrease. This trend can be explained by the decrease in overall enthalpy which takes place when cholesterol is increased, as the mixture requires less energy for the melting event to occur upon increased cholesterol levels as represented in figure 3.6.

Table 3.1: Effect of varying cholesterol content on the melting onset temperatures of the surfactant blend mixtures. Runs 6, 2 and 5 are represented from the design of experiments table where the MPG and DCP are kept constant.

	Run Order	Molar Ratio MPG:Chol:DCP		Melting Onset (°C)	Melting Enthalpy (J/g)	Crystallisation Enthalpy (J/g)	Reheat Enthalpy (J/g)
N1	1	5 : 2 : 0	Mean	74.94	164.6	96.25	136.63
			SD	0.40	6.5	18.83	6.48
N2	7	5 : 3 : 0	Mean	73.46	147.8	44.22	106.38
			SD	0.65	12.9	3.35	6.98
N3	9	5 : 4 : 0	Mean	73.29	129.5	33.78	100.73
			SD	0.44	4.8	3.82	2.19
N4	6	5 : 2 : 1.5	Mean	74.17	175.3	67.01	85.24
			SD	0.49	3.6	2.78	7.05
N5	2	5 : 3 : 1.5	Mean	73.54	139.0	42.43	60.01
			SD	0.74	8.8	11.47	14.87
N6	5	5 : 4 : 1.5	Mean	71.98	145.5	33.60	46.29
			SD	0.80	8.4	7.92	4.71
N7	11	5 : 2 : 3	Mean	73.69	168.9	64.75	92.05
			SD	2.07	19.6	6.83	14.12
N8	4	5 : 3 : 3	Mean	73.63	170.3	83.52	111.03
			SD	0.89	10.5	9.05	8.30
N9	3	5 : 4 : 3	Mean	73.50	159.4	67.47	95.34
			SD	0.67	10.6	16.30	16.54
N10	8	5 : 3 : 1.5	Mean	71.16	153.6	44.21	55.25
			SD	0.62	5.5	1.94	4.49
N11	12	5 : 3 : 1.5	Mean	73.64	167.9	59.78	79.84
			SD	0.87	5.9	3.05	6.84
N12	10	5 : 3 : 1.5	Mean	72.17	157.1	52.36	67.42
			SD	0.69	6.5	2.76	4.76

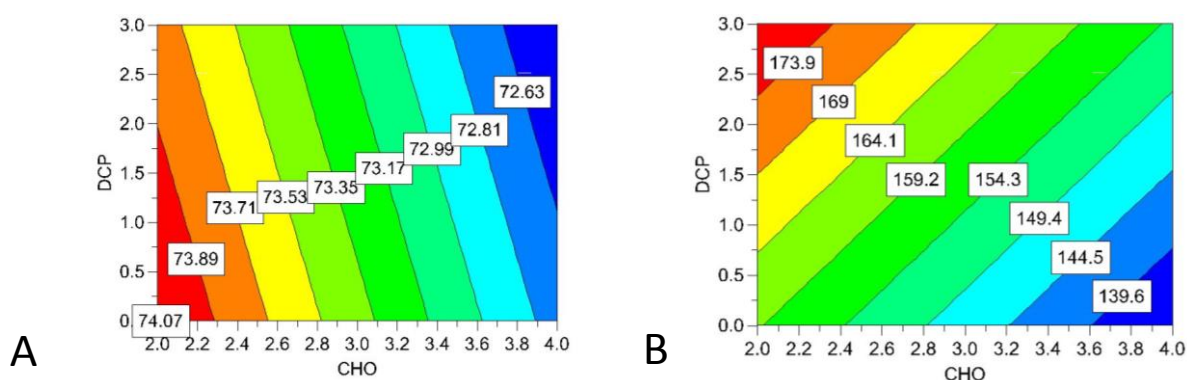


Figure 3.6: Contour plots showing the thermodynamic effects of DCP and cholesterol upon melting. **A)** The effect of cholesterol and DCP on the onset of melting (°C) and **B)** the effect of cholesterol and DCP on the melting enthalpy (J/g) of the mixture. MPG surfactant kept constant ratio of 5 for each ratio of DCP and cholesterol varied (Scan rate = 10 °C/min).

Table 3.1 shows that as the cholesterol ratio is increased from a ratio of 2 to 4 in runs 6, 2 and 5 respectively the onset of melting determined by DSC decreases from 74.17 ± 0.49 °C to 71.98 ± 0.80 °C respectively. This trend is also represented in Figure 3.6A, which shows the overall effect of cholesterol based on all of the formulations tested. Cholesterol has an onset melting point of 148 °C (Figure 3.4A) and as a result in the presence of MPG and DCP the total mixture is able to melt at 72-95 °C (Figure 3.4A). The cholesterol itself does not melt; however, it becomes miscible with the molten MPG/DCP mixture. Studies by Brostow and Datashvili, (2007) show that melting point depression of low density polyethylene occurs upon addition of different ratios of melamine formaldehyde indicating miscibility between the two components (Brostow and Datashvili, 2007). When analysing the combined melting of MPG and DCP a single melting peak with an onset temperature of 72.98 ± 0.17 °C and enthalpy of 204 J/g is obtained. By analysing the individual melting onsets and the heat energy required; DCP has a melting onset of 74.23 °C with a heat enthalpy of 222.6 J/g and MPG also has a very similar melting onset of 74.93 °C and a heat enthalpy of 194.8 J/g. The melting onsets of both are very similar and when used in ratio together at (5:1 MPG:DCP respectively) the melting onset reduces to 72.98 ± 0.17 °C with enthalpy of 204 J/g. Therefore, when the two surfactants are used in combination the melting onset temperature is reduced; however, the enthalpy of melting remains average of the two components. This can be explained in figure 3.4B, where the DCP shows a small transition at 43.34 °C and this event being the cause for interdigitation between MPG and DCP hence the lowering of the melting point. This is very common when using multiple component blends that two solids will co-melt together and cause a melting point depression with a broadening of the melting peak (Rice et al., 2011, Tran et al., 2004). For example, Tran et al, (2004) demonstrated that with the addition of just 2 mol

% cholesterol to DSPC liposomes both the transition temperature was depressed and enthalpy values resulting in broader melting peaks (Tran et al., 2004).

In the case of the 3 component mixture being investigated in these studies, Figure 3.6B, shows that as the DCP content is increased, the melting enthalpy increases suggesting more energy is required to melt the DCP. The DCP requires the highest melting enthalpy of 222 J/g and hence increasing the concentration will require more energy to melt. There is no difference in onset of melting observed as the structure still remains the same, but due to the increased DCP content the energy required to complete the melt increases. The incorporation of cholesterol at a ratio of 2 with MPG (5) and DCP (1) as shown in Figure 3.6 B, shows that the maximum heat enthalpy required to melt the mixture was 164 J/g (Melting onset 73.9 °C, Figure 3.6A) hence the inclusion of cholesterol has lowered the heat enthalpy further than that of the MPG:DCP (5:1) mixture whilst maintaining the melting onset. A further increase of cholesterol to a ratio of 4 with MPG:DCP (5:1) results in an enthalpy of melting at approximately 135 J/g which is considerably lower than that of the MPG:DCP mixture (204 J/g). This reduction in enthalpy has lowered the melting point onset further of the mixture to 72.26 °C. When the cholesterol content is increased the heat enthalpy required to break the bonds and allow for melting of the mixture decreases. Figure 3.4B, shows a thermogram of the mixture with a scan of cholesterol and DCP overlaid. The cholesterol shows a melting event at 35.66 °C which has an end point of approximately 60 °C suggesting that some of the cholesterol is possibly already in a molten state. As previously mentioned the lowering of melting point of a MPG and DCP mixture is caused by the DCP pre-melting event which associates itself within the MPG and DCP matrix resulting in bond weakness upon heating meaning that melting takes place at a lower temperature.

DSC studies by Othman et al, (2011) on the thermal effects of addition of corn starch to polyvinyl alcohol (PVOH) blends demonstrate that the addition of corn starch enhances the miscibility of the PVOH due to the molecular interactions which occur between the two components (Othman et al., 2011). Thus, lowering the melting point temperature by disrupting the crystalline structure of the PVOH as the addition of corn starch causes the enthalpy for melting to decrease in addition to reducing the melting temperature of the blend. Hence, the addition of cholesterol further enhances this miscibility process as the mixture requires even less enthalpy for melting due to the addition of cholesterol. The cholesterol content also influences the enthalpy for melting and the transition temperature upon formed vesicles. Eg, Cho et al, (2007) show that increasing cholesterol content within phosphatidylcholine liposomes broadens and reduces the melting onset and transition temperature and that this lipid to lipid interaction in the bilayer is dependent on the cholesterol content within the bilayers (Cho et al., 2007). The increase in cholesterol ratio results in a greater surface area of cholesterol within the mixture and interferes with the MPG and DCP lipid matrix which results in interference with the crystalline structures hence further reducing the enthalpy of melting which is shown in Figure 3.6B.

In conclusion, the thermogram in Figure 3.4A, shows that complete melting of the blend occurs between 95-98 °C and that when melting these components a temperature range of 120 °C can be avoided as the mixture is able to melt at a lower temperature. The results obtained indicate that the high melting point cholesterol is miscible with the other two molten surfactants where they impact on the crystalline structure of cholesterol thus allowing the powder blend to be melted at less than 100 °C preventing degradation of the component mixture which was confirmed by TGA and hot-Stage microscopy.

3.3.3 Hot stage microscopy confirming results of DSC

Hot stage microscopy was used to confirm the observations and conclusions from the DSC analysis in section 3.3.2. The hot stage was set up in a similar way to the DSC in that the program start temperature and end temperatures were maintained in the same way; however, a slightly slower scan rate of 6 °C/ min were employed due to the resolution and accuracy of the recording software. Figure 3.7 shows two image reels, A and B, which both comprise of the three component mixture.

Figure 3.7A shows that at 111 °C crystals remain present on the glass slide, which are presumably cholesterol. These cholesterol crystals are not in contact with the molten MPG/DCP and thus would require the hot stage to go to 150 °C to complete the melt of those cholesterol crystals, which was confirmed by an individual scan of cholesterol. However in Figure 3.7B the lipids were layered with MPG was placed on the bottom, followed by DCP and then cholesterol. The result shows that just before the complete melt (T_{max}) at 80 °C there are still crystals present and that complete melting occurs at 81 °C which corresponds to the DSC thermogram of the mixture. This result confirms that the cholesterol is able to dissolve into the molten MPG/DCP mixture and that this acts as a solvent for the cholesterol to dissolve in. Hence, when manufacturing at a large scale the order of addition of the components could increase melting efficiency by enabling melting to take place at lower temperatures, in turn preventing degradation of the surfactants at high temperatures. This idea of co-solvency to aid in melting point depression was also observed by Grandelli et al, (2012) where the melting point of the drug Piroxicam was significantly depressed when mixed with carbon dioxide (CO₂) or mixtures of CO₂ with co-solvents such as ethanol (Grandelli et al., 2012).

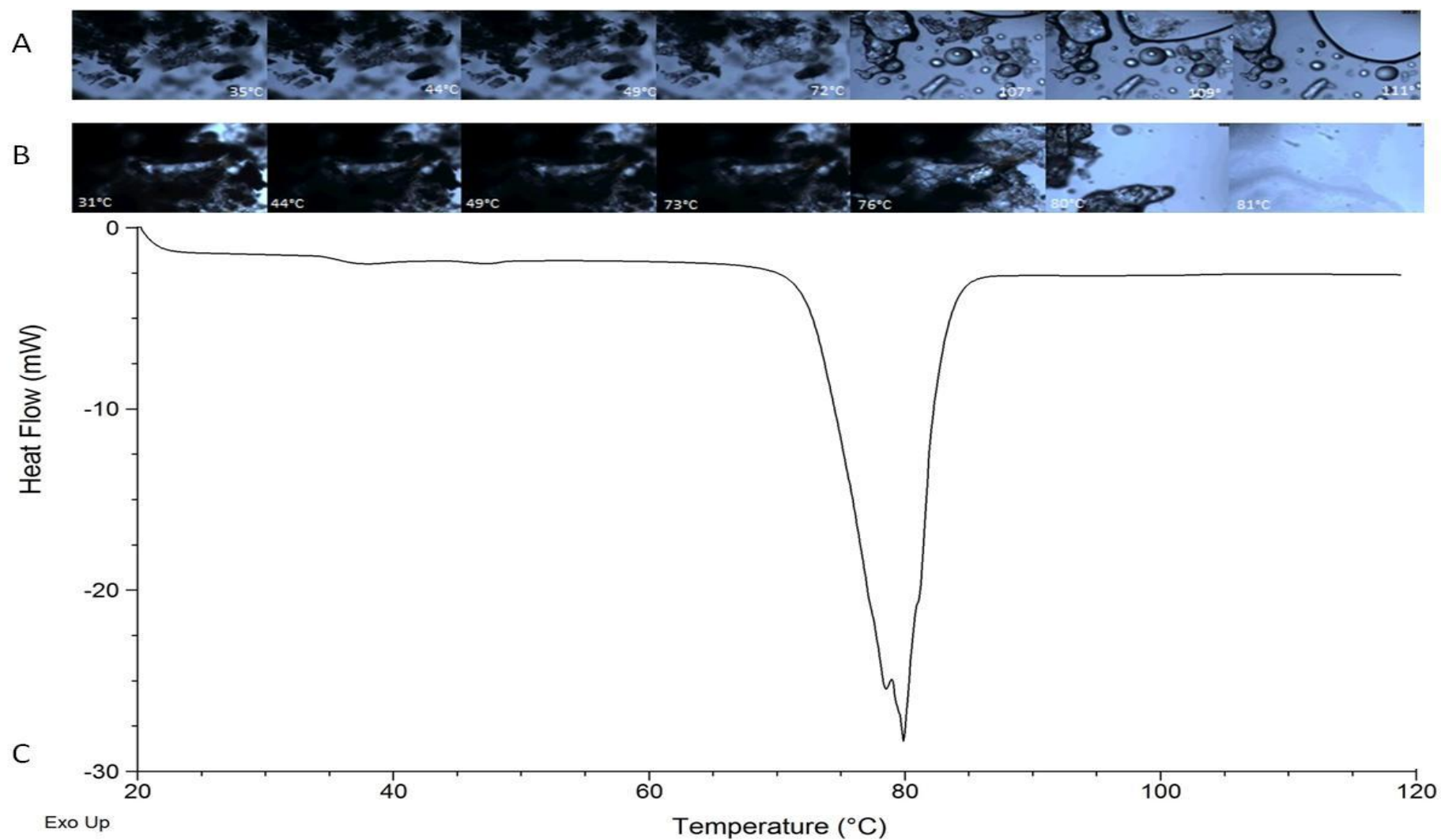


Figure 3.7: DSC thermogram of the surfactant mixture showing corresponding microscopy. A) MPG:Chol:DCP placed onto a glass slide randomly, B) Ordered mixture with MPG placed on bottom followed by DCP followed by cholesterol (Scan rate = 6°C/ min).

3.3.4 Langmuir monolayer isotherms

Before preparing the vesicles Langmuir monolayer studies were carried out to investigate how the surfactants interact with each other, potentially giving an insight into their interfacial properties with an aqueous subphase. The monolayer isotherm for the individual surfactants and the mixture of the surfactants is shown in Figure 3.8. Table 3.2 represents the values of the study showing the deviations for all experiments as $n=3$ and it is observed the deviation from the ideal extrapolated area at zero pressure is minimal of 0.9 % which is in line with the ideal extrapolated area.

The scan of MPG (Figure 3.8) shows that the surfactant isotherm exhibits several phase changes prior to collapsing when the surface pressure is increased. At 65 - 50 $\text{\AA}^2/\text{molecule}$ there is a gaseous phase, followed by a liquid expanded and gaseous phase from 50 - 35 $\text{\AA}^2/\text{molecule}$. At 35 - 22 $\text{\AA}^2/\text{molecule}$ a liquid condensed/ liquid expanded phase is present followed by a short liquid condensed phase to 20 $\text{\AA}^2/\text{molecule}$ where a sharp solid phase up to 16 $\text{\AA}^2/\text{molecule}$ is reached where the surface collapse pressure of $51.6 \pm 1.5 \text{ mN/m}$ is reached. In terms of cholesterol (Figure 3.8) the phase changes upon increasing surface pressure differ to those of the MPG surfactant. The cholesterol starts from a gaseous phase from 40 - 35 $\text{\AA}^2/\text{molecule}$ followed by liquid condensed phase from 35 - 30 $\text{\AA}^2/\text{molecule}$. After 30 $\text{\AA}^2/\text{molecule}$ sharp solid phase occurs up to 27 $\text{\AA}^2/\text{molecule}$ at a surface collapse pressure of $46.6 \pm 0.5 \text{ mN/m}$. Upon studying the DCP lipid, the isotherm is very similar to the cholesterol molecule where a gaseous phase exists between 50 - 40 $\text{\AA}^2/\text{molecule}$ until a liquid condensed phase is achieved between 40 - 36 $\text{\AA}^2/\text{molecule}$ until a final collapse pressure is reached of $53.8 \pm 0.9 \text{ mN/m}$ at 34 $\text{\AA}^2/\text{molecule}$.

The mixture of 5:4:1 molar/weight (308.5 mg) ratio of MPG:Chol:DCP respectively was prepared and the experiment was carried out in the same way as the individual components. The mixture exhibits a gaseous phase from 38 - 31 Å²/molecule where a liquid condensed phase occurs at 31 - 28 Å²/molecule followed by a sharp solid condensed phase at 24 Å²/molecule with a collapse pressure of 51.9 ± 0.1 mN/m (Figure 3.8). When extrapolating the mean molecular area at zero surface pressure for the mixture, based on the molecular weights and ratios of the components the ideal extrapolated molecular area was calculated to be 28.3 ± 1.5 Å²/molecule. Upon completion of the experiment the mixture resulted in an ideal extrapolated mean molecular area of 29.19 ± 0.19 Å²/molecule which showed no significant difference based on the ideal experimental calculated values. This shows that when the surfactants are in the 5:4:1 ratio of MPG:Chol:DCP they are forming uniform monolayers and the dispersion within the monolayer of the surfactants is even around the trough.

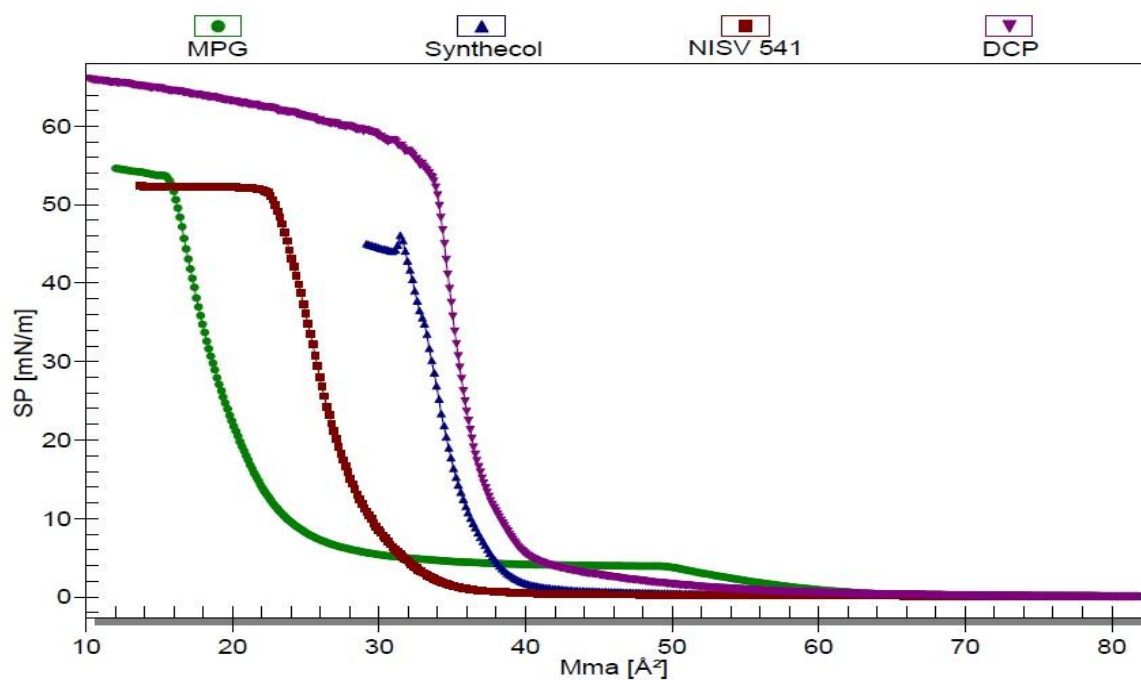


Figure 3.8: Langmuir monolayer isotherms of the individual components and the corresponding mixture 5:4:1 (MPG:Chol:DCP). All experiments were carried out in triplicate and at 20 °C.

Table 3.2: The experimental and ideal extrapolated mean molecular area and surface area compression pressure of mixed and pure monolayers at the air/water interface (at 20 °C). MPG, Chol, DCP and a mixture of ratio 5:4:1 (MPG:Chol:DCP respectively). All results are represented by n=3.

Component	Extrapolated area at Zero pressure (Å ² /molecule)	Ideal Extrapolated area at Zero pressure (Å ² /molecule)	Deviation from Ideality (%)	Collapse pressure (mN/m)
MPG	21.2 ± 0.4	-	-	51.6 ± 1.5
Synthecol	37.1 ± 0.4	-	-	46.6 ± 0.5
DCP	37.5 ± 0.5	-	-	53.8 ± 0.9
Mixture (5:4:1)	28.3 ± 1.5	29.19	0.9	51.9 ± 0.1

Langmuir isotherms are reviewed extensively by Moghaddam et al, (2011) where cholesterol and DCP, as used within this study, are shown under compression to move from a gaseous state to a liquid condensed state, and upon further contraction of the barriers forms a solid phase, until the monolayer of the surfactants collapses (Moghaddam et al., 2011a). The small molecules, cholesterol and DCP (without branching acyl chains), in this study show highly condensed surface pressure monolayer isotherms which are in line with studies carried out by Shah and Schulman (1968) (Shah Dinesh and Schulman Jack, 1968). However, the MPG isotherm shows different properties to those of Chol and DCP: where in Figure 3.8 the data shows that with the MPG surfactant there are more phases with a larger gaseous phase that occurs which includes a liquid expanded and gaseous phase prior to achieving a liquid condensed phase, which is common for saturated single chain molecules.

The cholesterol collapse pressure and extrapolated mean molecular area at zero pressure (46.5 ± 0.5 mN/m and 37.1 ± 0.4 Å²/molecule at Zero pressure; Table 3.2) are in line with previous studies (Dynarowicz-Latka et al., 2003, Slotte, 1992, Florence, 1981). For the combination of MPG:Chol:DCP (5:4:1 molar/ 308.5mg ratio), the ideal calculated mean molecular area is 29.19 Å²/molecule and the experimental value for the mixture is 28.3 ± 1.5 Å²/molecule (Table 3.2) suggesting that the surfactants are forming a

uniform monolayer and that they have equal properties where no one surfactant is more rigid or dominant over the others which is often seen with cholesterol. Furthermore, the presence of unsaturated fatty acyl chains or cholesterol has shown to liquefy DCP monolayers when mixed together and when applying the additivity rule of working out deviation from ideal molecular area, the monolayers of cholesterol-DCP follow the rule and show no deviation (Shah Dinesh and Schulman Jack, 1968). The cholesterol incorporation within the bilayer of high transition lipids has been shown to increase bilayer planarity affecting the bilayer packing density allowing uniform vesicles to form which become smaller and spherical and are better suited for laser diffraction (van Hal et al., 1996). However, the presence of cholesterol within low or no transition lipids such as phosphatidylcholine has shown to increase membrane mechanical rigidity and cohesiveness thus increasing the stability of liposome vesicles by reducing the permeability of the bilayers in the gel liquid state (McMullen et al., 2004, Kirby and Gregoriadis, 1980). The ability of cholesterol to interact with surfactants to reduce their transition temperature can also be studied using DSC. Liposomes were prepared with and without cholesterol and analysed by DSC (Figure 3.9). The Hydrophobic tails of the DSPC liposomes crystallise into a rigid-crystalline phase hence upon heating the tails become flexible giving rise to a transition temperature (Moghaddam et al., 2011a, Taylor and Morris, 1995). The DSPC scan in figure 3.9, represents one clear distinct transition peak with a smooth baseline which is an indicator of a pure liposome sample with no impurities segregated within the plane of the membrane hence a good indicator of the quality of the liposome sample prepared (Biltonen and Lichtenberg, 1993).

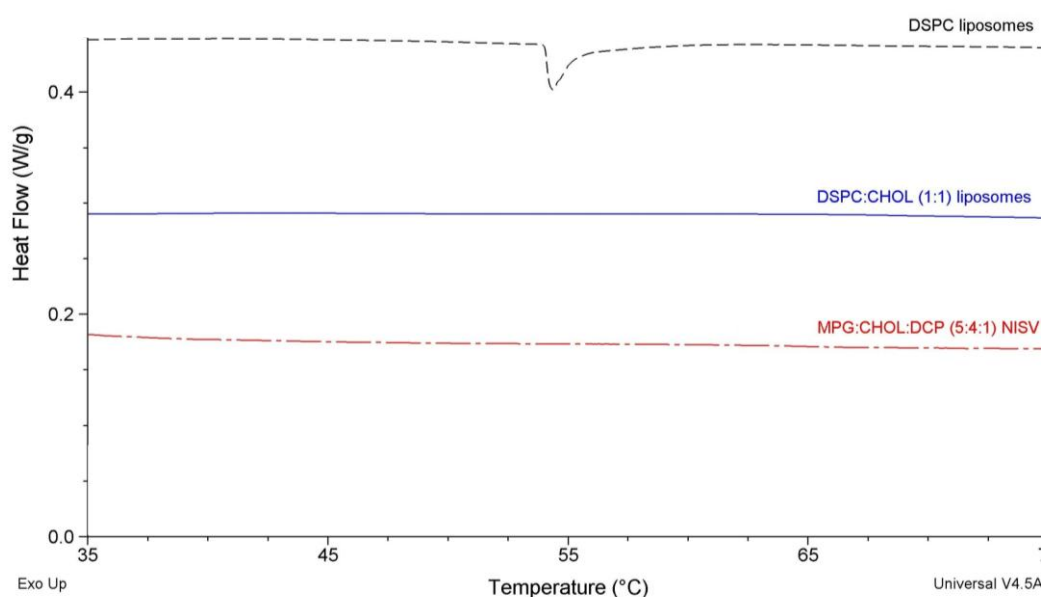


Figure 3.9: DSC thermogram of liposomes and niosomes. DSPC and DSPC:cholesterol liposomes prepared by lipid hydration were analysed for phase transition. A niosome preparation of 5:4:1 (MPG:Chol:DCP) shows no transition. Isotherm shows exothermic going up hence melting peaks are down the aqueous buffers were used as the reference (Scan rate = 10 °C/min samples run in triplicate).

The incorporation of cholesterol within the formulation allows the cholesterol to uniformly spread between the monolayers and insert itself between the lipids or surfactants hence preventing crystallisation of the hydrophobic tails. The presence of increasing cholesterol within lipid bilayers are to broaden and reduce the enthalpy of the liquid crystalline and gel phase eliminating it entirely at 50 mol % cholesterol (McMullen et al., 2004, Demel et al., 1972, Gregoriadis and Perrie, 2001, Oldfield and Chapman, 1972). Zuidam et al, (1995) further confirm that high mole fractions of cholesterol within lipid based vesicles remain physically stable and resist hydrolysis due the abolishment of the gel-liquid crystalline phase transition (Zuidam et al., 1995). Thus, our study confirms that the presence of cholesterol within the niosome vesicles results in an abolished transition temperature which was observed when analysed by DSC (both 95 °C and 120 °C preparations) as presented in Figure 3.9, thus indicating that the niosome vesicles should be physically stable and resist hydrolysis.

3.3.5 Stability study initial characteristics

As a result based on the information obtained about the surfactant mixtures by DSC, Hot stage Microscopy, TGA and Langmuir, vesicles were produced at two temperature ranges with an oil bath either set between 95-98 °C or 120-125 °C and upon manufacture the formed niosomes were investigated to elucidate if lower temperatures could be adopted in their manufacture. Within this study, the effect of temperature and storage were analysed by determination of vesicle size, zeta potential, suspension pH and by light microscopy. All of the formulations were tested on day 0 and a control study of empty niosomes alongside niosomes formulated with the model antigen, OVA, was carried out. The average vesicle size, surface charge and entrapment for the stability study in section 3.3.5 is represented in table 3.3 and the confirmation of the vesicles is visualised by the light microscopy images presented in figure 3.10.

Table 3.3: Initial vesicle size, zeta potential and antigen entrapment for the stability study. OVA niosomes n=6, empty niosomes n=3.

	Temperature (OVA NISV)		Temperature (Empty NISV)	
	95 °C	120 °C	95 °C	120 °C
Size (µm)	6.15 ± 0.1	6.52 ± 0.5	6.25 ± 0.04	6.58 ± 0.25
Span	1.73 ± 0.08	1.73 ± 0.05	1.81 ± 0.02	1.76 ± 0.01
Zeta (mV)	-63.23 ± 9.51	-61.69 ± 8.36	-81.48 ± 9.39	-80.17 ± 9.55
OVA entrapment (%)	23.51 ± 2.05	23.31 ± 1.18	-	-

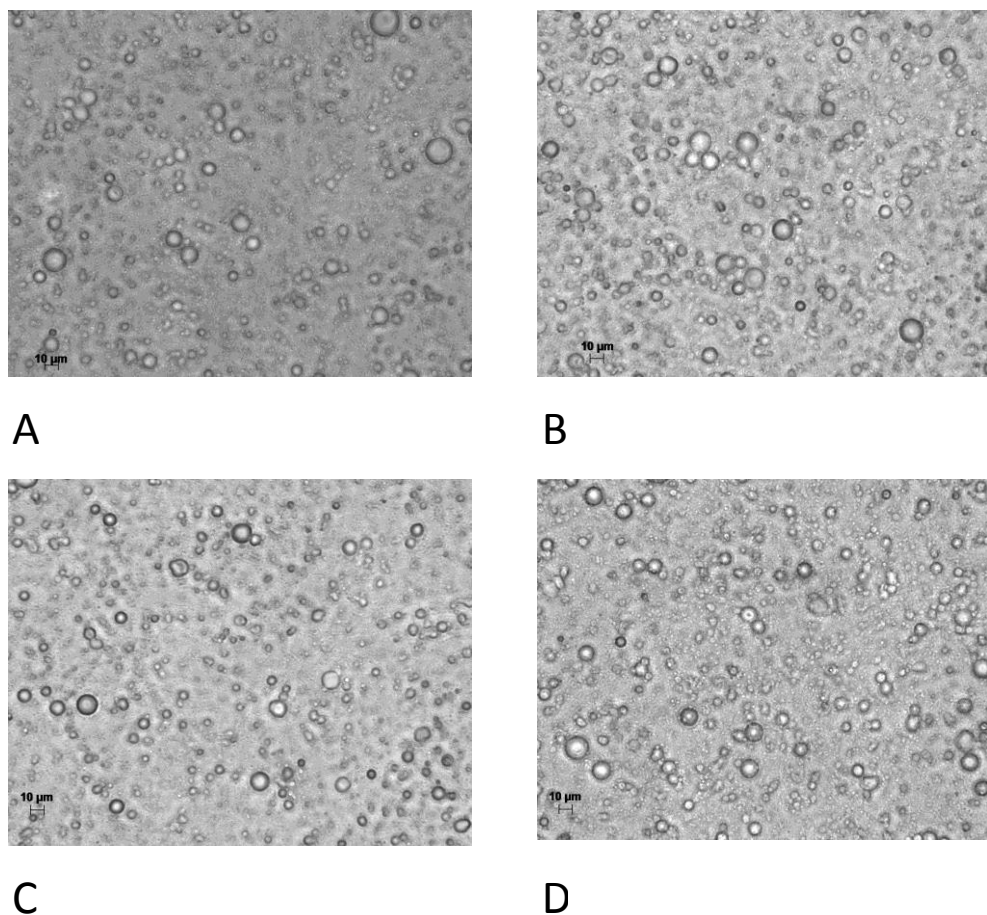


Figure 3.10: Light microscopy images of the niosome formulations with and without OVA at T=0. **A)** Preparation 95 °C empty, **B)** Preparation 120 °C empty, **C)** Preparation 95 °C OVA loaded and **D)** Preparation 120 °C OVA loaded. Scale bar represents 10 μm.

An average vesicle size of 6 μm is produced whether forming the vesicles by melting the surfactants at 95 °C or 120 °C (Table 3.3). As an initial observation no significant differences are present in terms of vesicle size when reducing the melting temperature of the surfactant mixture (Table 3.3). In terms of surface charge, zeta potential is the nearest experimental approximation of surface potential as both the surface potential and stern potential are difficult to measure. Thus the zeta potential is the potential at the surface of the shear plane which is measured by electrophoresis and is a good indicator of stability of colloidal suspensions or emulsions. The zeta potential is measured to understand aggregation and dispersion of vesicles within the formulation giving an insight into stability requirements. In terms of stability, the more negative the zeta

potential, the more likely the formulation is to be stable as the charged vesicles will repel each other hence avoiding aggregation (du Plessis et al., 1996). The initial zeta potential readings are presented in table 3.3 which are highly negatively charged indicating good stability (Freitas and Müller, 1998). The optical light microscopy images represented in figure 3.10 confirm the vesicle size distributions of the formulations alongside the zeta potential values mentioned in previous sections. The images show that the vesicles are well dispersed around and no aggregates are visible. The distribution of vesicles from large to small vesicles are visible on the images and are represented by the span values in Table 3.3.

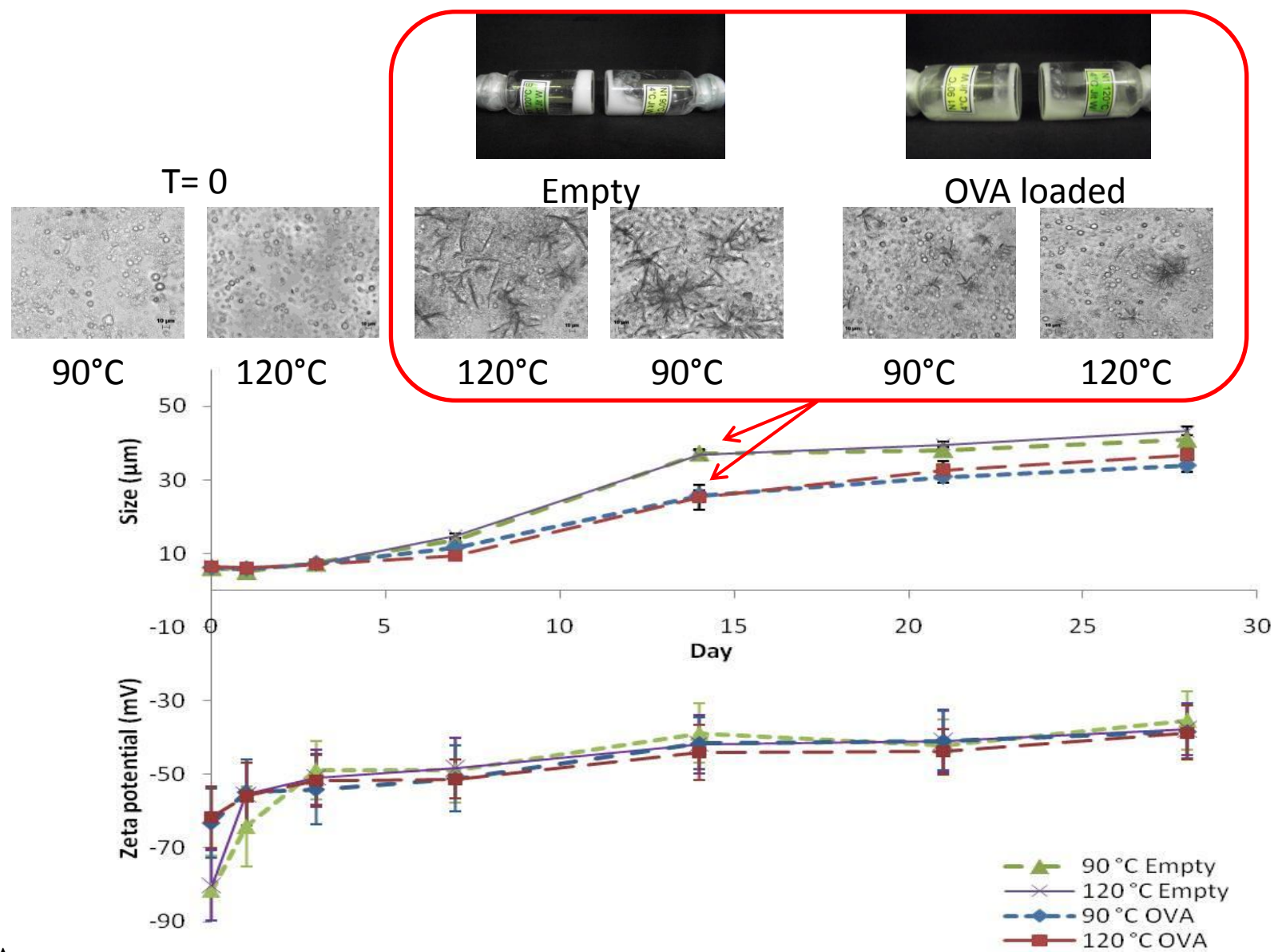
The OVA entrapment in the niosomes was calculated by a direct method using the ninhydrin assay mentioned in section 3.2.7. The ninhydrin assay is used as the presence of surfactants do not interfere within the assay in comparison to the Bradford, Lowry and Pierce Bicinchoninic Acid (BCA) assay (Brewer et al., 1995). Results of the entrapment studies show that the vesicles produced when the surfactants are initially melted at 95 °C have an entrapment of 23.5 ± 2.05 % (Table 3.3) and the vesicles produced with initial melting at 120 °C have an entrapment of 23.3 ± 1.18 %. Although the state of the protein is unknown, for vaccines confirmation is less important; however, it can be confirmed that the antigen is present and entrapment shows consistency between the two initial melting temperatures of the surfactants and is reproducible (n=6).

3.3.6 The effect of storage temperature on the stability of the niosome preparations

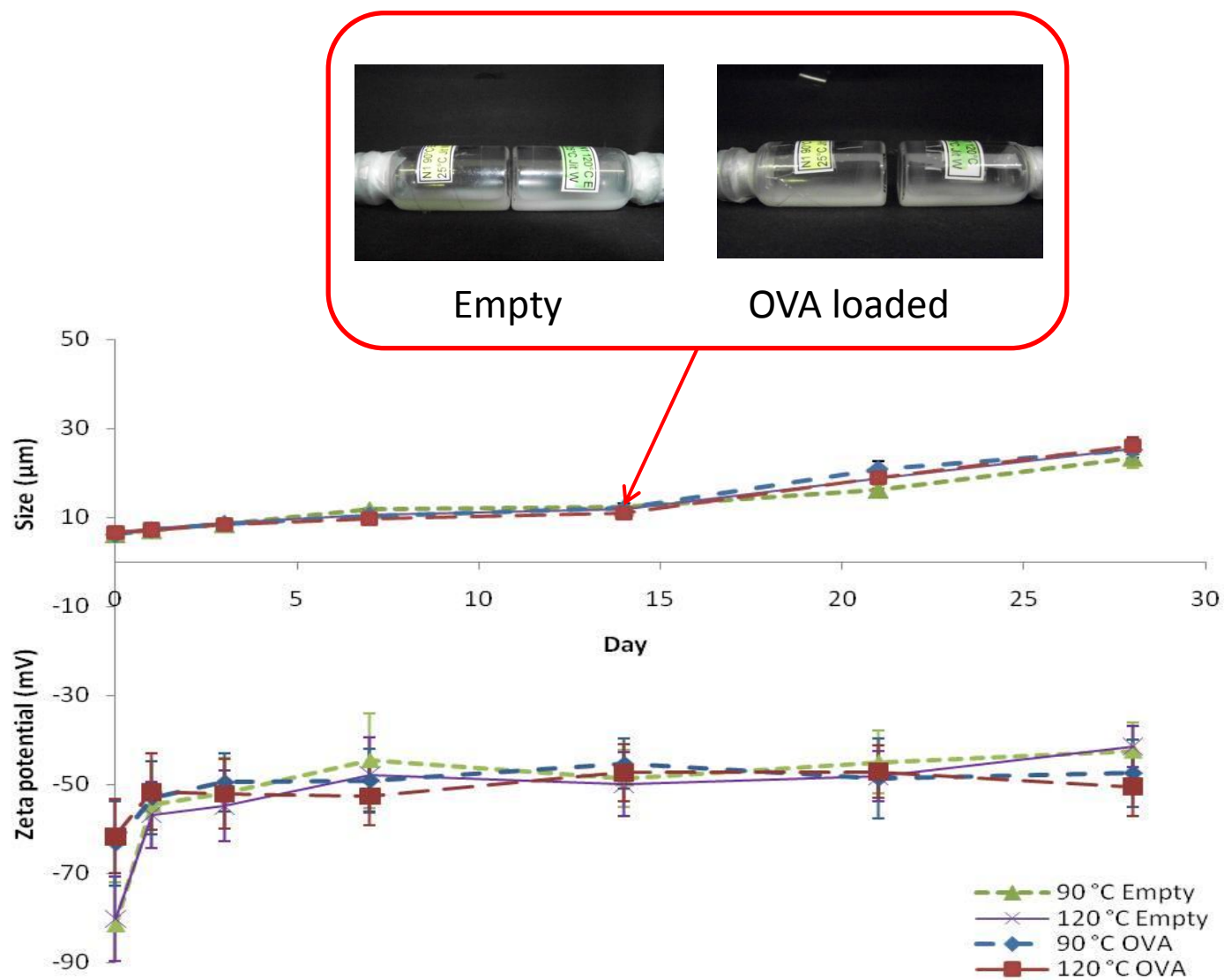
The aim of the stability study carried out on storage conditions is to see whether there are any significant differences in the vesicle characteristics by changing the initial

melting temperature of the surfactants. As observed in section 3.3.5 the pre-characteristic data shows no variable differences in the niosome formulations produced with either an initial melting temperature of 95 °C or 120 °C. The pH of all of the suspensions were all in the range of 9.47 ± 0.15 (results not shown). The formulations were prepared and subjected to different temperature storage conditions. Figure 3.11 represents the vesicle size and zeta potential for each storage temperature over a period of 28 days.

From the results the formulations stored at 4 °C (Figure 3.11A) increase in vesicle size at a faster rate than when stored at 25 °C or 40 °C (Figure 3.11B and C respectively). For example, the comparison of vesicle size of the OVA loaded vesicles at day 14 for the different storage temperatures varied greatly with niosomes having a VMD of $25.84 \pm 1.22 \mu\text{m}$ at 4 °C, compared to $11.95 \pm 1.09 \mu\text{m}$ and $10.07 \pm 1.18 \mu\text{m}$ at 25 °C and 40 °C respectively (Figure 3.11). The physical appearance of these formulations is represented in figure 3.11 which shows the OVA loaded vesicles at day 14 and at the different storage temperatures. The formulation at 4 °C shows signs of aggregation as there remains a sediment layer on the bottom of the vial when turned to its side whereas, the formulation at 25 °C or 40 °C do not show signs of sedimentation or aggregation at day 14. The niosomes kept at 4 °C after day 14 tend to show increased viscosity when physically agitating the vial in relation to the formulations stored at 25 °C and 40 °C which maintained fluidity.



A



B

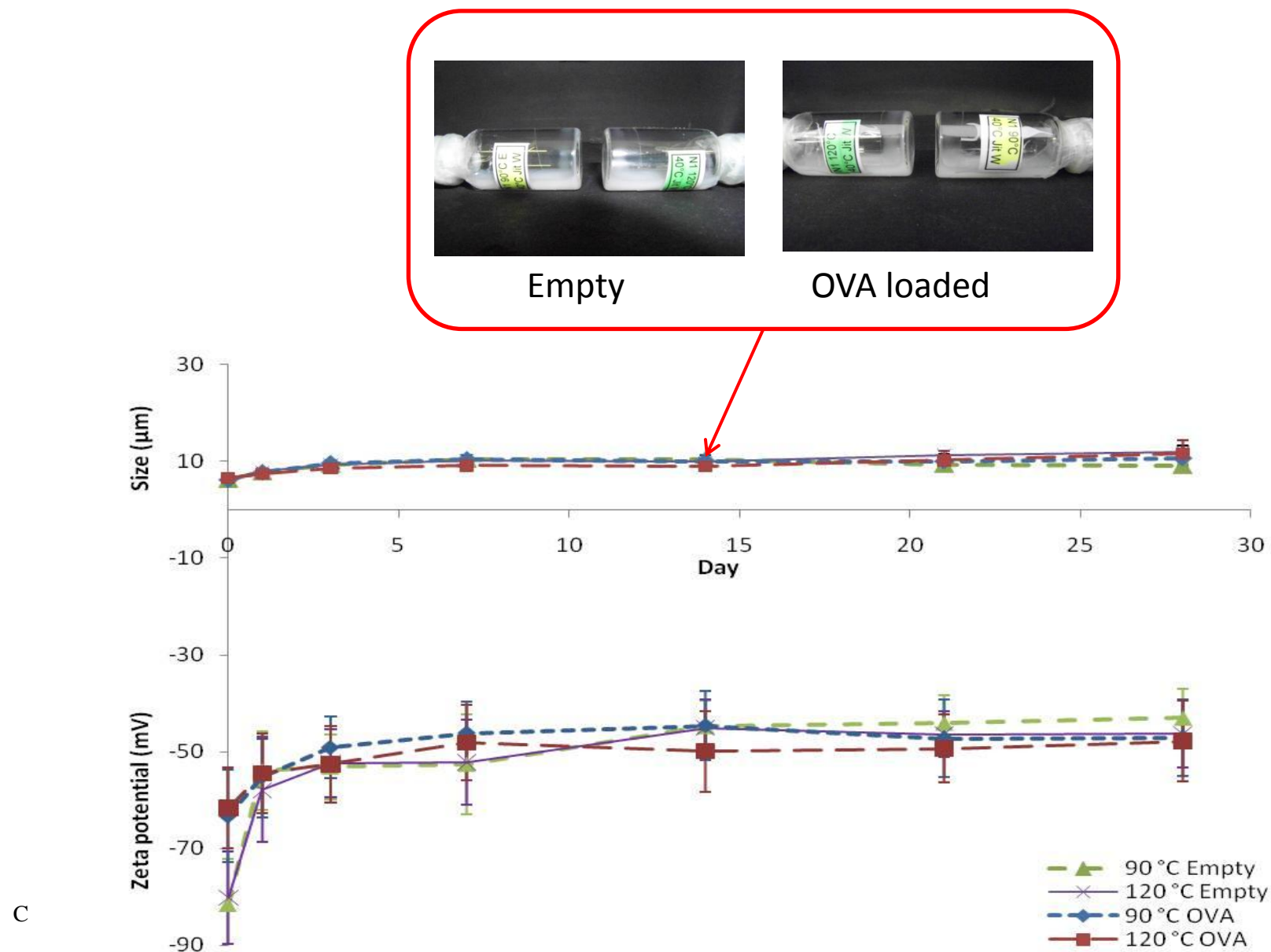


Figure 3.11: Characteristics of vesicle size and zeta potential of the formulations for a period of 28 days where the empty vesicles represent n=3 and the niosomes loaded with OVA represents n=6. Formulation vials out of the storage incubators to visually determine the appearance of the niosome preparations at day 14 **A)** 4 °C storage chamber showing corresponding light microscopy images, **B)** 25 °C storage chamber and **C)** 40 °C storage chamber.

Of each of the storage temperatures studied, the vesicles stored at 40 °C show better stability of the niosomes over a period of 28 days when compared to vesicles stored at 25 °C or 4 °C. The vesicles however within the formulation at 40 °C undergo morphological and structural changes into hexagonal shapes (Figure 3.12C/D). These shapes are visualised greater in the formulations stored at 25 °C (Figure 3.12A/B) from day 21 onwards hence suggesting that the niosomes when stored at higher temperatures are maintain their structure and shape for a longer time period.

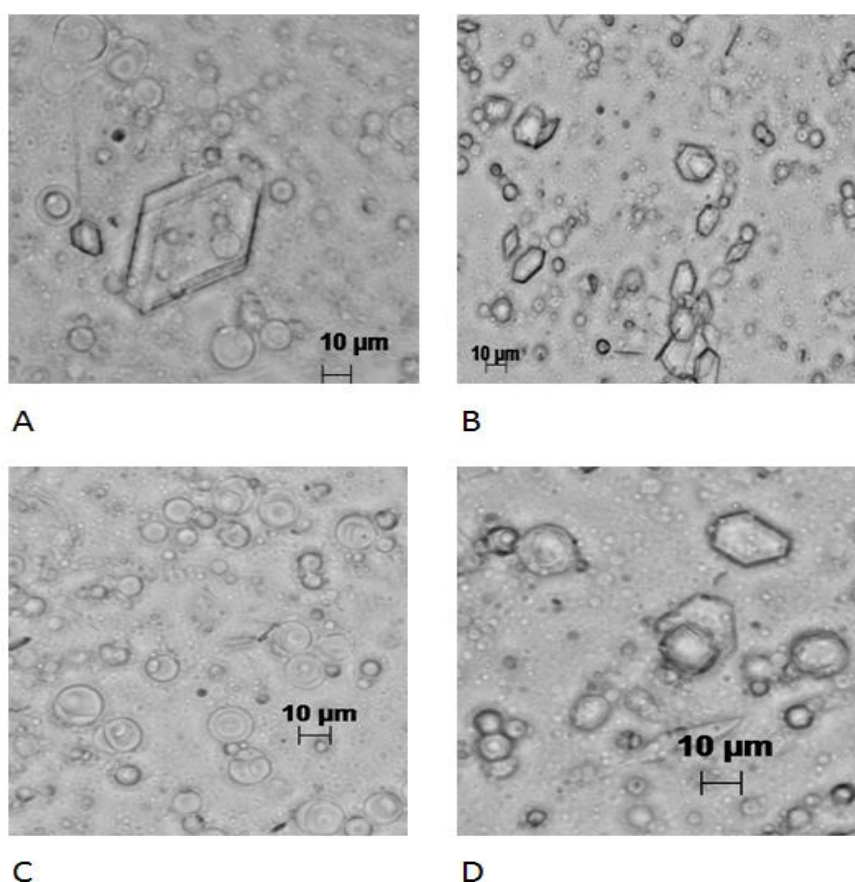


Figure 3.12: Day 28 formulations representing morphological rearrangement analysed by light microscopy at 40 x objectives. **A)** Empty niosome formulation stored at 25 °C, **B)** OVA niosome preparation stored at 25 °C, **C** and **D)** OVA niosome preparation stored at 40 °C, Scale bar represents 10 µm in all images and taken on a 40x objective.

When analysing the formulations prepared at 95 °C and 120 °C they showed the same properties in terms of sedimentation at each storage temperature (Figure 3.11) hence indicating that the initial melting temperature of the surfactant blend has little

implication on the physical properties of the formulations. The increased rate of sedimentation and destabilisation can be related to the slight differences in zeta potential of the formulations at the different storage temperatures (Figure 3.11). At 4 °C the zeta potential varies and heads towards the -30 mV range which results in destabilisation of the vesicles hence leading to aggregation and sedimentation. The zeta potential of the formulations stored at 25 °C and 40 °C show zeta potential of -50 mV with the deviations reaching -40 mV and still exhibit good stabilisation of the vesicles. Studies by Du Plessis et al, (1996) also show that over an extensive period of time at 4 °C and 25 °C storage of positively charged multi lamellar liposomes resulted in aggregation and increase in the vesicle mean diameter upon reduction in zeta potential (du Plessis et al., 1996). In addition, Uchegbu and Florence (1995) show that the presence of a charged lipid is vital in preventing aggregation when subjecting the vesicles into an isotonic solution (Uchegbu and Florence, 1995).

The DCP used within this study provides the net anionic charge to the vesicles hence resulting in a negative zeta potential (Chupin et al., 2002). The inclusion of a negatively (DCP) or positively charged (stearylamine) within vesicle bilayers stabilises the vesicles against fusion, aggregation and agglomeration (Barakat et al., 2009, Jadon et al., 2009). Studies show that optimum zeta potential should be greater than 60 mV for full electrostatic stabilisation for a positively charged moiety (Heurtault et al., 2003). Hence for a negatively charged vesicle, a highly negative charge of -60 mV would provide full electrostatic stabilisation. The zeta potential results of the vesicles to be used in the stability study in Table 3.4 are highly negative hence, are well dispersed within the suspension and are less likely to undergo aggregation hence showing excellent stability (Figure 3.10). The ‘empty’ vesicles show a greater negative charge to the vesicles containing OVA which could be due to the free OVA masking the

charge of the vesicles. In addition, the OVA could be inserting itself within the bilayer also masking the charge of the vesicle as studies by Chupin et al, (2002) have shown (Chupin et al., 2002). However, whilst not significantly different, the group sizes within these studies shows that there is a trend.

When comparing the formulations in figure 3.11 it is visible that the empty formulations at 4 °C show a faster rate of aggregation in relation to the formulations containing OVA. This trend is only observed at 4 °C and not at the other two storage temperatures. There is a possibility that due to the drop in surface charge which is from the DCP this may be an indicator of DCP leaving the vesicles and thus causing the aggregation and breakdown of the vesicles. Furthermore figure 3.11 confirms that there is a difference between the empty formulations and the OVA loaded niosomes at 4 °C. Figure 3.11 A at 4 °C shows that the formulations are very viscous and practically sediment to the bottom of the vial in relation to the OVA loaded vesicles in figure 3.11 B, which still show signs of fluidity. These samples were examined via light microscopy (Figure 3.11) where two sections of the same sample were screened to show that there are regions where there are intact vesicles in addition to regions where there are clusters or aggregates of degraded vesicles. At day 14, the empty vesicles show greater clusters and regions where the niosomes have disintegrated and are forming needle like structures.

Previous studies by Arunothayanun et al, (1999) on polyhedral niosomes have shown that niosomes are sensitive to temperature (Arunothayanun et al., 1999). The study shows that when niosomes held below the transition temperature form polyhedral niosomes which resemble the vesicles in this study (Figure 3.12). Furthermore, Arunothayanun et al, (1999) discuss that when polyhedral niosomes are present the viscosity of the samples increase in relation to the more spherical niosomes due to the

presence of rigid gel shapes (Arunothayanun et al., 1999). This effect can be related to our study where fewer polyhedral shapes are visible within the formulations held at 40 °C and are represented by the minimal vesicle size change (Figure 3.11C) as these remain less viscous when stored at higher temperatures (40 °C). Whereas, the niosomes stored at room temperature show an increased presence of polyhedral shapes as the storage time increases so does viscosity in relation to the samples stored at the higher temperatures (40 °C) which is in line with the studies carried out by Arunothayanun et al, (1999). As a result, vesicles stored over time start to undergo bilayer transformations and change shape resulting in the increased viscosity of the formulations. At 4 °C (Figure 3.11) there are no signs of polyhedral niosomes or any shapes of this description. The formulations in figure 3.12 show the viscosity of the samples at the low temperature of 4 °C is at its greatest and shows destruction of the vesicles when analysed visually (Figure 3.11).

Although the vesicles were initially prepared at 50 °C, resulting in spherical niosomes, the cold storage temperatures over the period of 28 days has shown vesicle destruction, whereas the vesicles stored at higher temperatures show increased stability and retention of spherical vesicles for a longer period of time. Furthermore, studies on vesicles by Vangala et al, (2006) also show that liposome vesicles prepared from DDA showed an increase in vesicle size when stored at 4 °C over a period of 7 days, eventually leading to precipitation of the vesicles by day 28. In addition, the incorporation of TDB in the DDA liposome vesicles showed that over a period of 28 days there was no significant increase in vesicle size (Vangala et al., 2006). The OVA present within the formulations seems to stabilise the vesicles from breakdown at 4 °C (Figure 3.11B) for a longer period of time up to day 14; however, at day 28 the empty vesicles and the OVA loaded vesicles look the same under the microscope and the

evident size result shows that the size distribution between the niosomes with and without OVA end up to be the similar (Figure 3.11).

3.4 Conclusion

Overall, the purpose of this study was to determine whether the mixture of surfactants can be initially melted at a lower temperature than stated in the literature to prevent any degradation occurring of the surfactants at the elevated temperatures. The study shows that the use of analytical techniques such as DSC and TGA confirm that the surfactant mixture is able to melt at a lower temperature hence, vesicles were prepared and tested for physico-chemical properties over a period of 28 days and compared to vesicles prepared at the literature values. Results show that when melting the lipids at 95 °C or 120 °C there are no significant differences to vesicle size, zeta potential or pH. Hence, niosome vesicles can be prepared at a lower melting temperature thus preventing degradation of surfactants and in turn allowing the cost of manufacturing to be lowered due to the decrease in melting temperature. In regards to future preparations, as there is no significant difference in physico-chemical characteristics between the two preparation temperatures formulations will be melted at 120 °C to speed up the melting process as the degradants formed had no impact upon the immune response (confirmed by VBI). In terms of temporary storage, niosome vesicles should preferentially be stored in the fridge for a maximum of three days and stored at either 25 °C or 40 °C for a maximum of 24 hours to see no significant change in vesicle size. However, it is recommended that for long term storage stability, the niosomes should preferentially be lyophilised in order to maintain stability during storage.

Chapter 4

Development of a novel thermostable vaccine by exploiting non-ionic vesicles

Patent related to this Chapter:

Anderson D, Kirchmeier, M, Campbell, M, Wilkhu J, Perrie Y. Methods for preparing vesicles and formulations produced therefrom. International PCT Patent Application No: PCT/US12/21389. Filed January 13.2011.

4.1 Introduction

All of the currently licensed vaccines are sensitive to heat, thus requiring the use of cold chain environments for storage during transport. On the other hand, many vaccines such as hepatitis B are sensitive to freezing temperatures (Braun et al., 2009, Chen et al., 2009a). Therefore, vaccines are stored with a temperature range specified at 2-8 °C to maintain their potency. However, protecting the vaccines from excessive heat and freezing during transport and storage can be difficult in developing countries (Matthias et al., 2007, Nelson et al., 2007, Techathawat et al., 2007) and studies have shown that temperature deviations for vaccines are more likely to occur after delivery of the vaccines to storage centres, or immunisation clinics, rather than at the production sites (Setia et al., 2002, Matthias et al., 2007). For example, a study in Hungary confirmed that 4 % of the vaccines were compromised due to the summer heat and 38 % compromised by the winter freeze (Lugosi and Battersby, 1990). Other issues related to consequences of compromised vaccines include, insufficient training, power shortages and human errors (Setia et al., 2002). As a result, the consequences of failures during cold chain transportation/storage can result in the increase of immunisation costs and local vaccine strategy plans due to vaccine wastage and inadequate protection of those unidentified products.

Therefore, the thermostability of vaccines is critical in providing effective prevention of disease and currently the approaches to overcome the issues of vaccine stability include the use of particulate delivery systems. Thermal stability of components required to produce vaccines in solution is strongly influenced by factors such as pH, osmolarity, ionic strength and the presence of excipients (Brandau et al., 2003). Currently non-ionic surfactant vesicle carrier systems, such as niosomes or bilosomes, are being employed to encapsulate/ associate vaccine antigens and to promote their effective delivery via the

oral route. However, the methods used to form these vesicles can require antigens to be heated to 60 °C or be subjected to several freeze thaw cycles to prepare the vaccine delivery systems (Conacher et al., 2001, Mann et al., 2009). This can have a major impact on the integrity of the vaccine; for example, Statens Serum India have shown that after three freeze thaw cycles toxoid vaccines such as Tetanus, Diphtheria and Pertussis lose up to 60 % potency and this freeze damage is sustained (Kartoğlu, 2006). Given that the potency of a vaccine can be decreased by exposure to excess temperatures it is essential to maintain vaccine components at their ideal temperatures for maximum potency (Plotkin and Fletcher, 1996, Galazka et al., 1998). Yet this can be costly, thus the development of a thermostable vaccine presents the ideal solution to this problem.

4.1.2 Aims and Objectives

Therefore, the aim of the work outlined within this chapter was to develop a novel method of preparing niosome vesicles which can protect the vaccine antigen against elevated storage conditions for a portion of its shelf life. This was carried out by preparing Fluzone associated niosome vesicles and after lyophilisation transporting them to Variation Biotechnologies (Canada) and determining if the developed method gives a response *in vivo* compared to a commercial vaccine (Fluzone). Upon determination of the ability of the proposed method of production to induce an immune response the niosome vesicles were to be subjected to storage at 40 °C and 4 °C for 3 months prior to *in vivo* administration to determine thermostability compared to the commercial vaccine and the current method of niosome production.

4.2 Materials and Methods

4.2.1 Surfactants

A range of non-ionic surfactant based bilayer vesicles were prepared by high shear homogenisation and analysed using several materials and methods. The surfactants used in the study were Monopalmitoyl glycerol (MPG) (Larodan Labs, Sweden), Synthecol (Chol) (Sigma Aldrich, UK) and Dicetyl Phosphate (DCP) (Sigma Aldrich, UK). The aqueous buffer sodium bicarbonate (Sigma Aldrich, UK) or phosphate buffered saline (PBS) was buffered to pH using a 1 M sodium hydroxide solution (Sigma Aldrich, UK) or 0.1 M hydrochloric acid (Fisher Scientific, UK).

4.2.2 Radio-labelling of H3N2 antigen

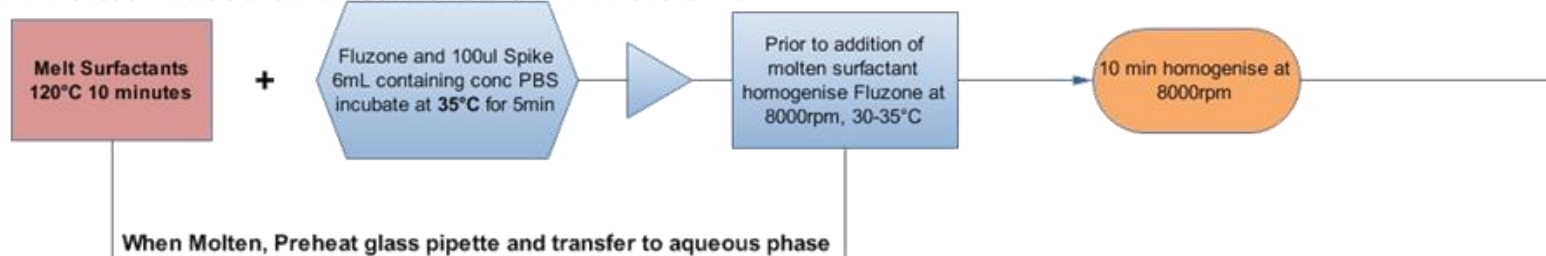
The H3N2 antigen (Immune Tech, USA) stock solutions were prepared as 100 µg of purified protein (1 mg/mL) in PBS stock and 40 µL (40 µg) placed into an iodination tube (Pierce Biotechnology) with 2 Mbq of ¹²⁵I (Perkin Elmer, Belgium) for 1 hour. A separation column was pre-prepared by firstly, heating Sephadex G75 (Sigma Aldrich, UK) beads for 3 hours at 90 °C and left to cool, during that time the column was prepared by placing glass wool at the bottom to prevent any Sephadex beads from falling through the column. The column was then packed using the pre heated and swollen Sephadex G75 prepared earlier. It is important that the gel remains hydrated with the PBS buffer which is used for labelling. It is essential to optimise the flow rate based on sample collection and then to match the sample volume collected to the correct protocol on the gamma counter.

Upon packing of the column, just above gel, the solution from the iodination tube was added to the top of the column and then rehydrated with buffer and samples were collected every 1 minute for 1 hour which results in the collection of 500 µL per vial.

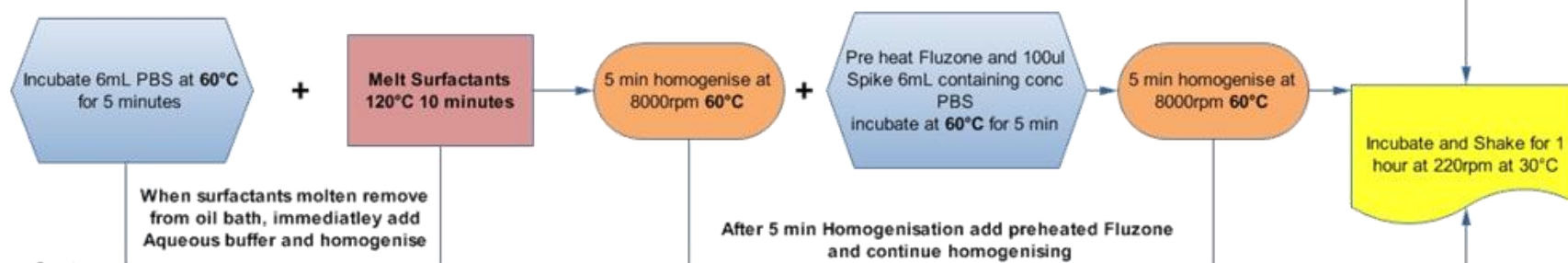
The vials were then analysed for ^{125}I content using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA) individually to prevent any backscatter that may occur from neighbouring vials. To confirm presence of protein in the vials a micro- BCA assay was carried out and used in parallel with the data obtained from the iodination of the protein.

4.2.3 Preparation methods of vesicles

VBI Inverted Based on Document # 3.1.14 Version.0



Strathclyde 3 step based on SOP: V5 NB08-02-100-AU1



Aston

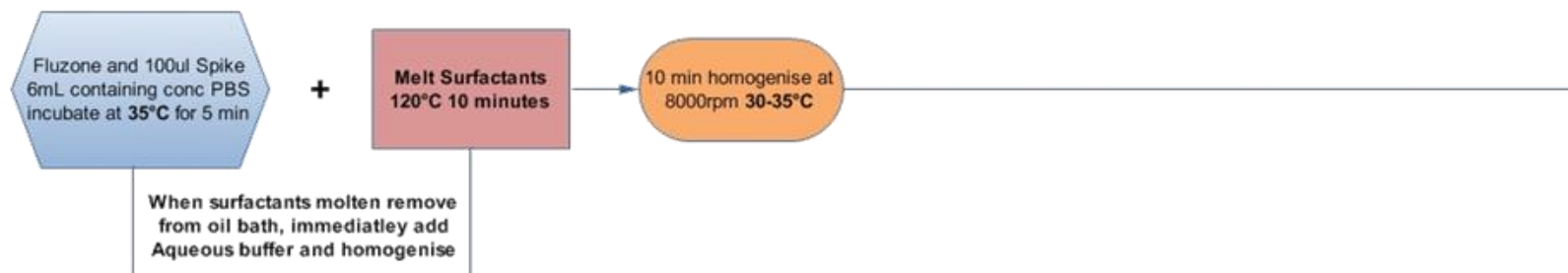


Figure 4.1: Summary of methods for preparing niosome vesicles as presented within the patent by Anderson et al, 2011.

4.2.3.1 Preparation of niosomes using Aston melt method

A 5:4:1 molar ratio of MPG, Chol and DCP was weighed out using a balance and placed into a 10mL glass beaker ensuring that no lipids stick to the sides of the beaker. Before melting the lipids, the aqueous phase was prepared in a 15 mL falcon tube. 25 mM sodium bicarbonate (pH 7.6) formed the bulk part of the aqueous phase, and radiolabelled antigen (Section 4.2.2) was placed in a heated water bath for 10 minutes at 30-35 °C. Hence this was to be known as the buffered antigen stock solution.

While the aqueous buffer was preheated, the beaker containing the lipids was placed into a hot oil bath (120-125 °C) and melted for 10 minutes. The beaker containing the molten mixture was removed from the oil bath and the buffered antigen stock solution was immediately added to this beaker and immediately homogenised at 8000 rpm at 30-35 °C. The mixture was homogenised for 10 minutes and left to shake and incubate at 220 rpm at 30 °C for 2 hours.

4.2.3.2 Preparation of niosomes using Strathclyde melt method

Niosomes were also prepared using a method first developed by researchers within Strathclyde university (Mann et al., 2004); a 5:4:1 molar ratio of MPG, Chol and DCP was weighed and placed into a 10 mL glass beaker. Before melting the lipids, the aqueous phase was prepared in a 15 mL falcon tube. 25 mM sodium bicarbonate (pH 7.6) formed the bulk part of the aqueous phase. The buffered solution was placed into a heated water bath at 60 °C. A 100 µL radiolabelled antigen was then placed in a heated incubator for 5 minutes at 37 °C. While the aqueous buffer was preheated, the beaker containing the lipids was placed into a hot oil bath (120-125 °C) and melted for 10 minutes with occasional swirling. The beaker containing the molten mixture was removed from the oil bath and the 25 mM sodium bicarbonate buffer is immediately

added to this beaker and then immediately homogenised at 8000 rpm at 60 °C for 5 minutes. Upon 5 minutes total homogenisation time the radiolabelled antigen was added to the homogenising suspension and homogenised for a further 5 minutes and then left to shake and incubate at 220 rpm at 30 °C for 2 hours.

4.2.4 Characterisation of vesicles

The size of the vesicles was determined using laser diffraction on a sympatec 2005 (Helos/BF) analyser. 20 µL aliquots of the vesicle suspension were diluted into the cuvette with 40 mL double distilled water. The zeta potential was measured in 1.5 mL double distilled water at 25 °C on a Zeta Plus Brookhaven Instrument. 20 µL of the bilosome suspension was mixed in 1.5 mL double distilled water and then analysed. The pH of the vesicle suspension was determined using a pH meter where the tip was placed into the vesicle suspension and left for a few minutes.

4.2.5 Quantification of antigen

For quantification of antigen association, ultra-centrifugation of the formulations was required to isolate antigen entrapped vesicles, from non-incorporated antigen. To achieve this, 300 µL aliquots of sample were diluted in a Beckman 3.9 mL Polo-allomer tube and filled up to the neck with double distilled water. The tubes were carefully heat sealed ensuring that there are no air bubbles present within the tube. The tubes were then placed accordingly in the TLN-100 rotor and centrifuged at 354,000 X g for 45 minutes at 4 °C with the acceleration set at 8 (Slowest) and the deceleration at coast. When centrifugation was complete, the tubes were opened and the supernatant removed. The sample was resuspended and the centrifugation stage repeated. After the second 'wash' the antigen loaded vesicles are then re-suspended with 100-200 µL of appropriate buffer and transferred all to a clean/labelled 1.5 mL eppendorf tube.

4.2.6 Separation of free antigen from associated antigen via gel separation

The Sepharose CL-4B solution was mixed in a ratio of 75:25 % ratio of Sepharose and PBS and shaken to ensure a homogenous mixture of the Sepharose CL-4B beads. Glass wool was placed into a 5 mL glass burette column where pre-swollen Sepharose CL-4B gel was packed into the column and PBS was allowed to run through the column for up to 1 hour to ensure successful packing of the gel. 300 µL of the radiolabelled niosome formulation was pre-counted on the gamma counter to obtain an initial count. The sample was then taken from the gamma vial and placed onto the top of the gel within the column and was then allowed to pass through and the column then rehydrated with PBS. The samples were collected at 0.25 mL per two minutes for a period of two hours. Radioactive counts were determined using the gamma counter and antigen association was calculated.

4.2.7 Trypsin digestion

Vesicles were prepared with radiolabelled antigen and an initial antigen association was calculated by centrifugation. Trypsinisation studies were carried out by incubating aliquots of the sample with 100 µg/mL trypsin for a period of 30 and 60 minutes at 37 °C to remove any adsorbed antigen. The samples were then centrifuged (Section 4.2.5) to determine surface adsorption of the antigen.

4.2.8 Lyophilisation of niosome vesicles

Lyophilisation was performed with the Virtis Advantage (Bio Pharma) freeze dryer. A 400 mM Trehalose solution was prepared and then added 1:1 to the niosome samples to be freeze dried and were then stored at -70 °C. The freeze drying protocol was set for primary drying to occur at -40 °C for 48 hours with a secondary drying cycle set at 20 °C for a further 10 hours with a condenser of -75 °C. The freeze drying cycle is

sufficient enough to thoroughly dry the formulations and prevent damage to the samples.

4.2.9 Direct Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA), was used to detect the presence of antibody in serum collected from mice. The direct detection method used a labelled primary antibody that reacts directly with the antigen. Direct detection could be performed with antigen that was directly immobilized on the assay plate or with the capture assay format. Diluted antigen in PBS was used to coat a 96 well plate using 100 μ L of solution followed by incubation at 2-8 °C overnight. 300 μ L of a diluted goat serum in PBS was used as blocking solution. A series of 2 fold dilution of the serum sample prepared in a blocking buffer was assayed in a 96 well plate to detect the antibody titre. The results were recorded at OD 450 nm and chart was prepared to detect the titres of the sample.

ELISA assay was performed on 96 well ELISA plates and were coated overnight at 4 °C with recombinant protein. The next morning the plates were washed with PBS containing 0.05 % Tween 20 and then blocked (1-3 h at 37 °C) with 10 % goat sera in PBS. After the incubation time, plates were washed six times in wash buffer (0.05 % Tween 20 in PBS). The starting dilution of the sample in 10 % goat sera in PBS was prepared and 2-fold serial dilutions were carried out. The sample and the standard were added to the 96 well ELISA plates and were incubated for 1.5 h at 37 °C. The plates were washed six times in wash buffer and incubated for 1.5 h at 37 °C with a 1/10000 dilution of a goat anti-mouse IgG-Fc HRP conjugated secondary antibody (Bethyl). The plates were washed six times and developed with 100 μ L of TMB substrate for 8 min.

100 μ L of TMB-Stop solution was added to stop the reaction. Absorbance was read at 450 nm with an ELISA plate reader (Bio-Rad).

4.3 Results and Discussion

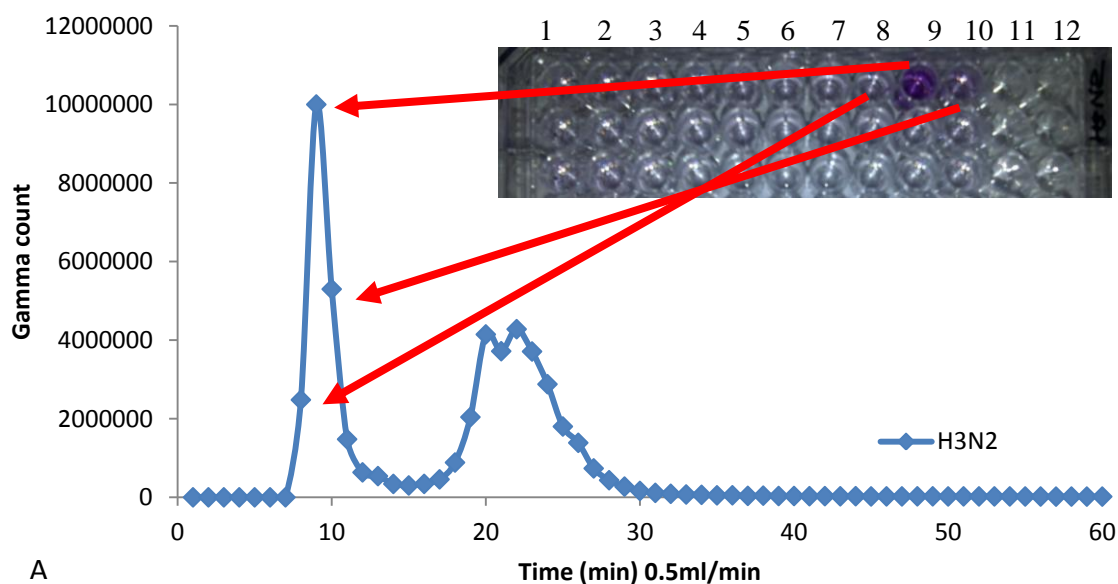
4.3.1 Development of Aston melt method

Due to problems of stability of vaccines at excessive heat or freezing temperatures, protection methods, such as association with particulate delivery systems and subsequent lyophilisation has resulted in increased shelf life protection of the vaccines. As previously mentioned, niosome vesicles have shown the potential to both improve antigen-specific immune responses and offer increased protection of vaccines (Brewer and Alexander, 1992, Walker et al., 1996, Murdan et al., 1999). However, the current patented methods of manufacture of niosomes require preparation temperatures of 60 °C (Figure 4.1; commonly referred to as the Strathclyde melt method) which can be detrimental to thermolabile vaccine antigens. As a result, within this chapter a method has been developed that can overcome this barrier and allow the use of thermolabile antigens to be incorporated within niosome vesicles. The new method proposed in figure 4.1 (referred to as the Aston melt method) has significant differences to the previously patented method in section 4.3.2.3 (referred to as the Strathclyde melt method). Firstly, the manufacturing temperature is reduced from 60 °C to 30-35 °C which in turn, should restrict degradation of antigen during the formulation process. Secondly, the antigen is added to the process at the start prior to vesicles being formed with the intention of increasing the amount of antigen entrapped within the vesicles therefore offering increased protection. To determine whether these changes offer differing properties, vesicles prepared using each of the two methods were compared for antigen loading and retention after trypsinisation and then subsequent in-vivo

immunisation studies. To determine entrapment the H3N2 antigen was firstly radiolabelled to determine quantification of antigen studies.

4.3.2 Radiolabelling of antigens

Fluzone is a trivalent vaccine comprising of three influenza antigens H1N1, H3N2 and Brisbane in the year 2009. All three of the antigens were radio-labelled with ^{125}I as outlined in section 4.2.2. Figure 4.2 shows the radiolabelling of A) H3N2 and B) Brisbane antigens where the radiolabelled antigen elutes first at approximately <7 mL (14 min) whilst free ^{125}I elutes > 10 mL. The presence of antigen within the first peak was confirmed with a micro BCA assay kit (which turned purple on the detection of antigen; Figure 4.2). The aliquots containing the antigen were pooled to form the stock solution for the subsequent studies.



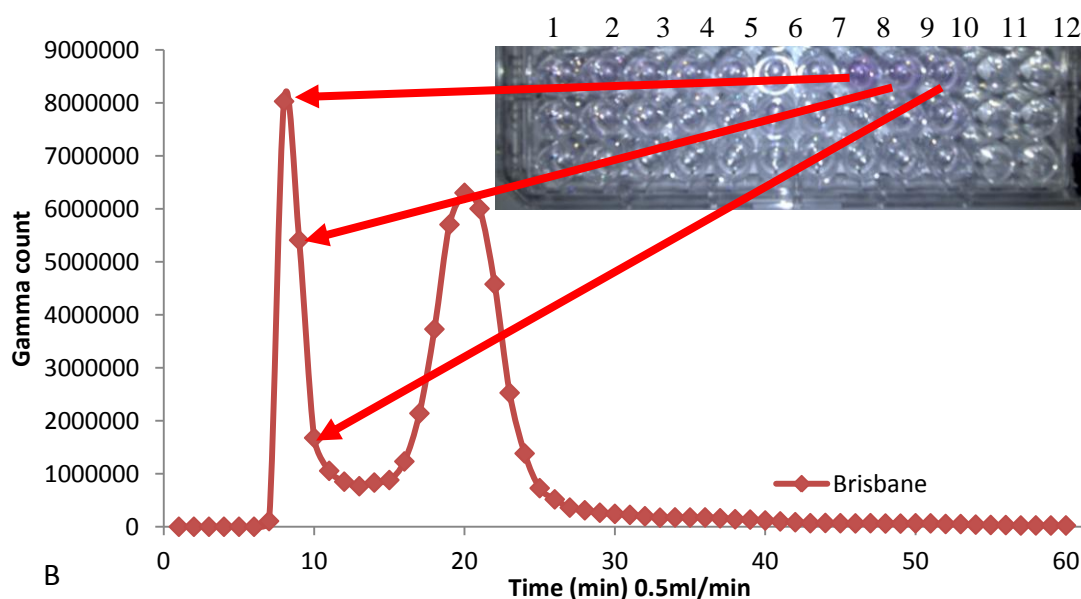


Figure 4.2: A) The radiolabelling of the H3N2 antigen showing two peaks indicating radiolabelled antigen and free ^{125}I with the confirmation of the antigen present in peak 1 wells 8-10 using a Micro-BCA assay kit. B) The radiolabelling of the Brisbane antigen showing two peaks indicating radiolabelled antigen and free ^{125}I with the confirmation of the antigen present in peak 1 wells 8-10 using a Micro-BCA assay kit.

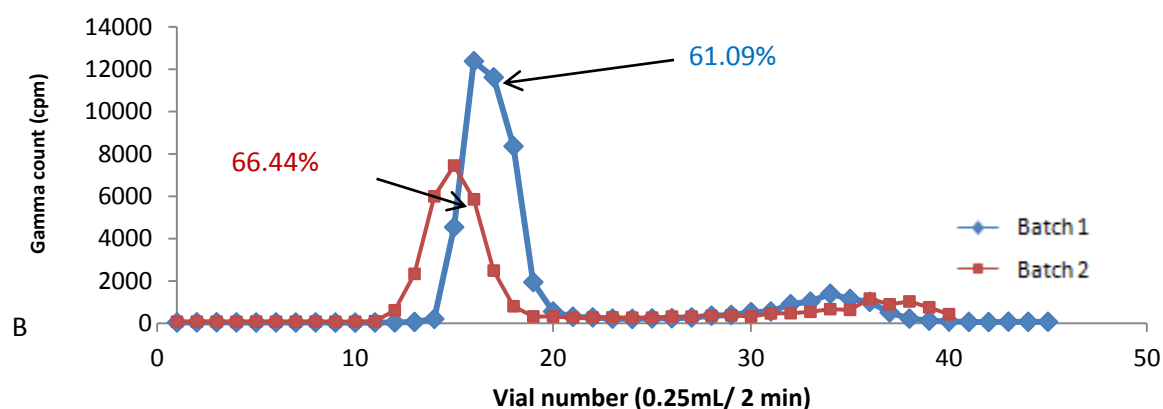
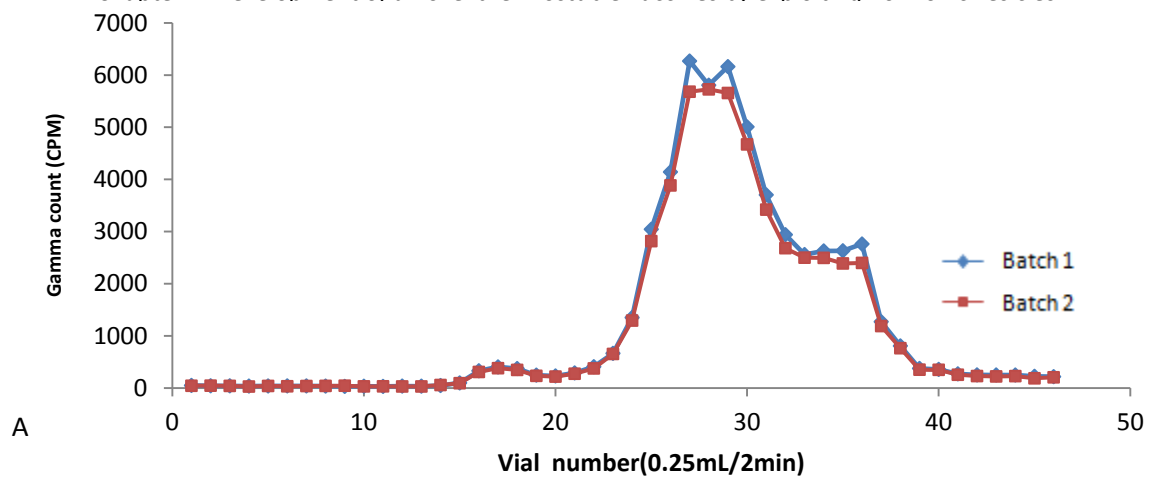
4.3.3 Validation of centrifugation protocol

To remove non-associated antigen from niosome formulations various methods can be adopted including centrifugation, column chromatography and dialysis. However, of these methods centrifugation offers the most convenient method for formulation screening of multiple formulations. Therefore to validate the use of centrifugation to remove non-associated antigen from niosomal carriers, studies initially focused on comparing the efficiency of non-associated antigen removal using centrifugation (Brewer and Alexander, 1992) and column chromatography using Sepharose CL-4B (Stuhne-Sekalec et al., 1991, Uchegbu and Florence, 1995). The columns used were prepared with Sepharose CL-4B as per protocol in section 4.2.6. The Sepharose CL-4B column has a bead diameter of 40-165 μm enabling the niosomes to pass through the column whilst delaying the elution of the free antigen (as this passes through the matrix of the gel). In all cases background values on the gamma counter were taken to be below 300 cpm. Initially 'free' antigen was passed through the column to identify its

elution volume. Independent batches of niosome formulations which were tested for antigen loading using column separation chromatography antigen loading compared to the corresponding association values obtained for the centrifugation method (Figure 4.3). Niosome vesicles were prepared as outlined by the VBI inverted melt method in figure 4.1.

Figure 4.3 A shows that the free antigen starts to appear from vial numbers 16-18 (4 to 4.5 mL), however as a small percentage ($< 1\%$) of the initial counts. This could be due to possible aggregates of the antigen, hence travelling through the sepharose column at a faster rate than the rest of the free antigen which appears as a broad peak from vial 23 (5.75 mL) onwards. A comparison of niosome-associated H3N2 via centrifugation and column separation was also determined by preparing niosome vesicles with the H3N2 antigen, where 300 μ L of the sample was counted and then passed through the column as in section 4.2.6. In addition to passing through the column another 300 μ L from the same batch of niosome vesicles was counted and association was determined using centrifugation as per protocol in section 4.2.5.

The results from both centrifugation and column separation show highly comparable results of antigen association with the niosome vesicles. Figure 4.3A shows that the free antigen starts to appear from vial number 23 (5.75 mL). In comparison, niosomes with associated antigen are eluted from vial number 13 to 18 (from 3.25 mL to 4.5 mL; Figure 4.3B and C). The data shows that the reproducibility between the processes is around 5 % and that the centrifugation procedure with the H3N2 antigen is valid with no indication that the free antigen is spinning down with the antigen associated niosomes at a process temperature of 30 °C.



D

	Centrifugation		Column Chromatography	
	Batch 1	Batch 2	Batch 1	Batch 2
free antigen recovered	14.85 %	16.39 %	-	-
antigen associated with niosomes	60.74 %	63.61 %	61.09 %	66.44 %

Figure 4.3: **A)** Determination of elution volume for free H3N2 antigen; **B)** niosome preparations incorporating the antigens H3N2; **C)** confirmation of niosome peaks using the vials after passing through Sepharose CL-4B column, and **D)** corresponding centrifugation antigen-loading results for H3N2.

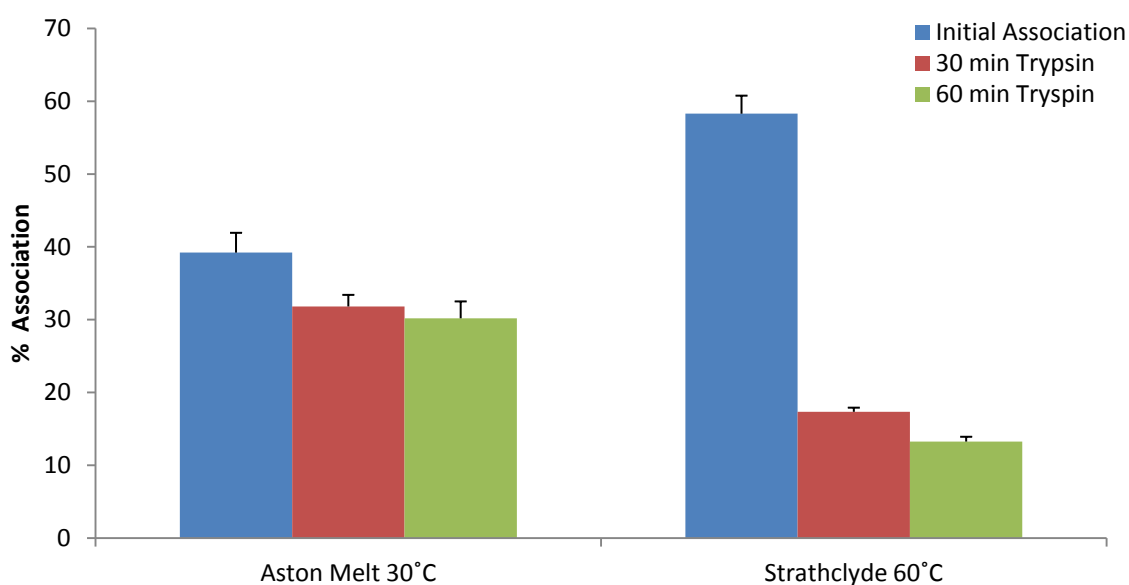
4.3.4 Initial association of antigen with vesicles and digestion with trypsin

Niosome vesicles containing Fluzone (which included radiolabelled H3N2 antigen) were prepared via the Strathclyde melt method and the new Aston melt method as per protocol in section 4.2.3. Results indicate that, whilst the vesicles are comparable in terms of their initial physico-chemical characteristics (Table 4.1), when comparing the initial association values between the two methods there is significant differences in antigen association with the Strathclyde melt method having significantly higher ($p < 0.01$; ANOVA with post-tukey test) association of antigen compared to the Aston melt method (Figure 4.4). However, upon digestion with trypsin for 30 and 60 minutes, the Aston melt method gave significantly higher antigen protection ($p < 0.01$) from degradation and in turn having greater antigen entrapped within the vesicles compared to the Strathclyde melt method (Figure 4.4).

After 30 minutes of trypsin exposure antigen-loaded vesicles prepared by the Strathclyde method (Section 4.2.3.2), antigen association dropped to $\sim 17\%$ and subsequently after 60 minutes trypsin exposure, antigen association dropped to $\sim 13\%$ from an initial association of $\sim 58\%$ (Table 4.2). This suggests that 45% of the niosome associated antigen was accessible to digestion, and that this antigen is surface bound given that trypsin is unable to cross vesicle bilayers (Kaur et al., 2012) (Table 4.2). In comparison, the Aston melt method, although initially less antigen ($39.21 \pm 1.59\%$) is associated to the vesicles compared to the Strathclyde melt method, trypsin digestion studies after 30 minutes show that more antigen ($31.82 \pm 1.59\%$) is entrapped within the vesicles thus offering significantly ($p < 0.01$) increased protection and recovery of antigen compared to the Strathclyde method ($17.35 \pm 0.57\%$; Table 4.2).

Table 4.1: Physicochemical characteristics of the niosome formulations prepared by either Aston melt or Strathclyde melt.

Niosome	Vesicle size (μm)	Zeta potential (mV)	pH
Aston melt	4.84 ± 0.16	-102 ± 12	7.4
Strathclyde melt	5.14 ± 0.11	-103 ± 21	7.4

**Figure 4.4:** Digestion of antigen with trypsin after 30 and 60 minutes incubation at 37 °C compared to initial association measured with radioactive counts (n=3).**Table 4.2:** Initial H3N2 antigen association and protection after trypsin digestion at 37 °C for 30 and 60 minutes.

Method	Initial % Association	30 min Trypsin	Protection %	60 min Trypsin	Protection %
Aston	39.21 ± 2.72	31.82 ± 1.59	82	30.19 ± 2.32	77
Strathclyde	58.29 ± 2.48	17.35 ± 0.57	30	13.26 ± 0.66	23

The differences in antigen retention between the two niosome preparation methods (Figure 4.4) could be due to difference in when antigen was added in the formulation process and the temperatures used. Using the Strathclyde method (Section 4.3.2.3) the

antigen is added to preformed vesicles thus potentially promoting increased surface adsorption of the antigen, whilst in the revised Aston method, the antigen is added during formation of the niosomes, which may promote higher incorporation within the vesicles. Secondly, the difference in temperatures between the two preparation processes which may influence and potentially denature the antigen thus changing niosome/antigen interactions. This effect has been demonstrated by Privalle et al, (2011), whereby a phosphinothricin acetyltransferase protein exposed to 60 °C was still detectable after exposure however, showed total loss in its enzymatic activity (Privalle et al., 2011). The overall increased recovery of antigen within the Aston melt method can be attributed to the addition of antigen within the formulation process. The antigen is present within the aqueous phase prior to the formation of the vesicles, thus, upon vesicle formation there is an increased potential for antigen to be incorporated within the vesicles compared to surface adsorption. This is comparable to previous studies where antigen was loaded onto the surface of MLV and compared to antigen entrapped within vesicles using the dehydration-rehydration method (Kaur et al., 2012). Within this study, cationic vesicles were used to deliver a TB sub-unit antigen and it was demonstrated that whilst initial loading of antigen was noted to be similar between both vesicles preparation methods, vesicles with antigen incorporated within vesicles offered higher antigen protection to trypsin digestion compared to liposomes where the antigen was surface bound (Kaur et al., 2012). Therefore from the data within Table 4.2 it can be seen that the Aston melt method offers increased antigen protection potentially due to enhanced entrapment of antigen within the vesicles, and this may be advantageous for antigen delivery.

4.3.5 Determination of immune response from Aston melt method

From the data shown so far, the Aston melt method has shown promise as a process for vesicles, resulting in increased antigen entrapment within niosome vesicles and offering increased protection of the antigen upon digestion with trypsin. Although having increased antigen retention and protection shows promise, it is important that these attributes facilitate production of an immune response. As a result, niosome vesicles were prepared via the Aston melt method with the trivalent Fluzone vaccine, subsequently lyophilised and reconstituted prior to administration intramuscularly to female Balb/c mice (6-8 weeks old). In general, an intramuscular dose of antigen to mice require the dose to be $1/10^{\text{th}}$ the human dose (45 μg). Therefore, the commercial vaccine was used as the control formulation and injected at 4.5 μg per dose compared to the prepared niosome vaccine which had a reduced 1.35 μg antigen per dose. The formulations were administered as 50 μL doses in the right quadricep of the balb/c mouse. Blood samples were taken at specific intervals and then immunological analysis was carried out by ELISA.

The physicochemical characteristics of the vesicles used for the immunisation study are presented in Table 4.3 where the vesicle size was 4.58 μm with a highly negative surface charge of -101 mV and a neutral pH. The consideration of pH is an important factor; Influenza antigens within a pH range of 7-9 show no loss of potency, however losses in antigen potency have been shown above and below this range (Brandau et al., 2003).

Table 4.3: Physicochemical characteristics of the niosome formulation

	Vesicle size (μm)	Zeta potential (mV)	pH
Aston melt	4.58 ± 0.12	-101 ± 17	7.4

Results from the ELISA experiments for the three strains (Figure 4.5) show that by incorporating Fluzone with the niosome vesicles, endpoint titres are increased and offer a stronger immunological response compared to the commercial Fluzone vaccine. When comparing the niosome preparation to the commercial Fluzone control vaccine, there were significant differences in the H1N1 strain and the B-Florida strain showing that the niosome vaccine offers stronger systemic immunity compared to the commercial Fluzone. However, there were no significant differences in immunity for the H3N2 strain.

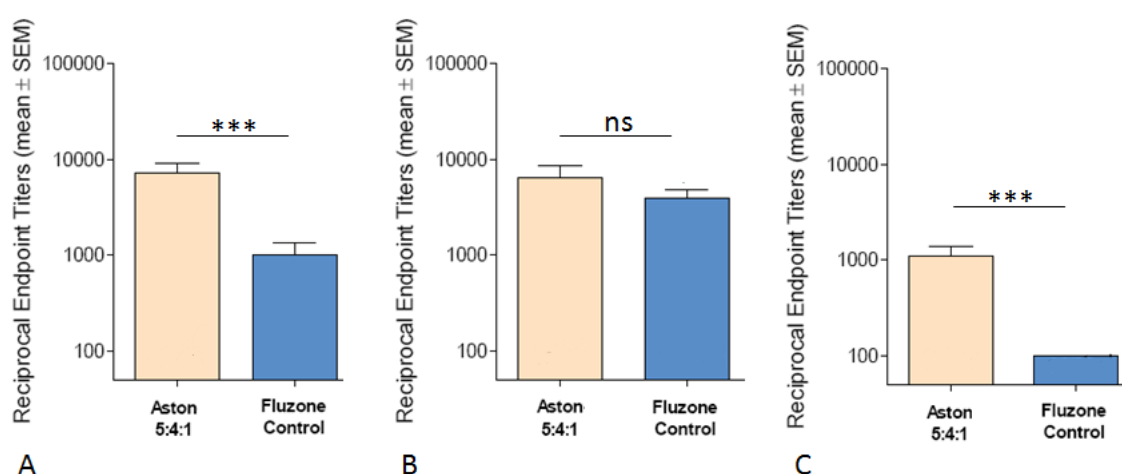


Figure 4.5: Reciprocal HAI endpoint titres of the trivalent Fluzone incorporated into niosome vesicles via the Aston melt method compared to the commercial Fluzone vaccine. **A)** H1N1 strain, **B)** H3N2 strain and **C)** B-Florida strain (n=5).

The study shows that the development of the Aston melt method is successful in inducing systemic immunity against influenza. Based on the results within figure 4.5, antigen entrapped/associated within niosome vesicles offers potentially increased systemic immunogenicity compared to the commercially available Fluzone vaccine. Furthermore, the niosome vaccine prepared, contained a much lower dose than the commercial vaccine when administered and still showed increased immunity, thus showing the potential of the niosome vesicles to act as a dose-sparing adjuvant. Studies by Moon et al, (2012) on the development of a malaria vaccine with the use of PLGA

nanoparticles have also demonstrated that conjugation of antigen to the nanoparticles results in a significant increase in serum IgG titres compared to the same free antigen dose. Moreover, upon significant reduction in antigen dose when conjugated to the nanoparticles, antibody levels produced were still significantly higher than the antigen only dose, thus showing that the use of carrier systems potentially allows a reduction in antigen dose to produce dose sparing vaccines whilst maintaining effective immunity (Moon et al., 2012). In addition, Wee et al, (2008) have shown that mucosal immunity can be enhanced by the use of H1N1 influenza antigen when administered with an ISCOMATRIX system containing cholesterol, phospholipids and saponins. Their study showed that pulmonary administration of a significantly dose reduced H1N1 antigen from 15- 0.04 μg in the ISCOMATRIX system showed serum antibody responses greater than or equal to the standard 15 μg unadjuvanted antigen dose when delivered subcutaneously (Wee et al., 2008).

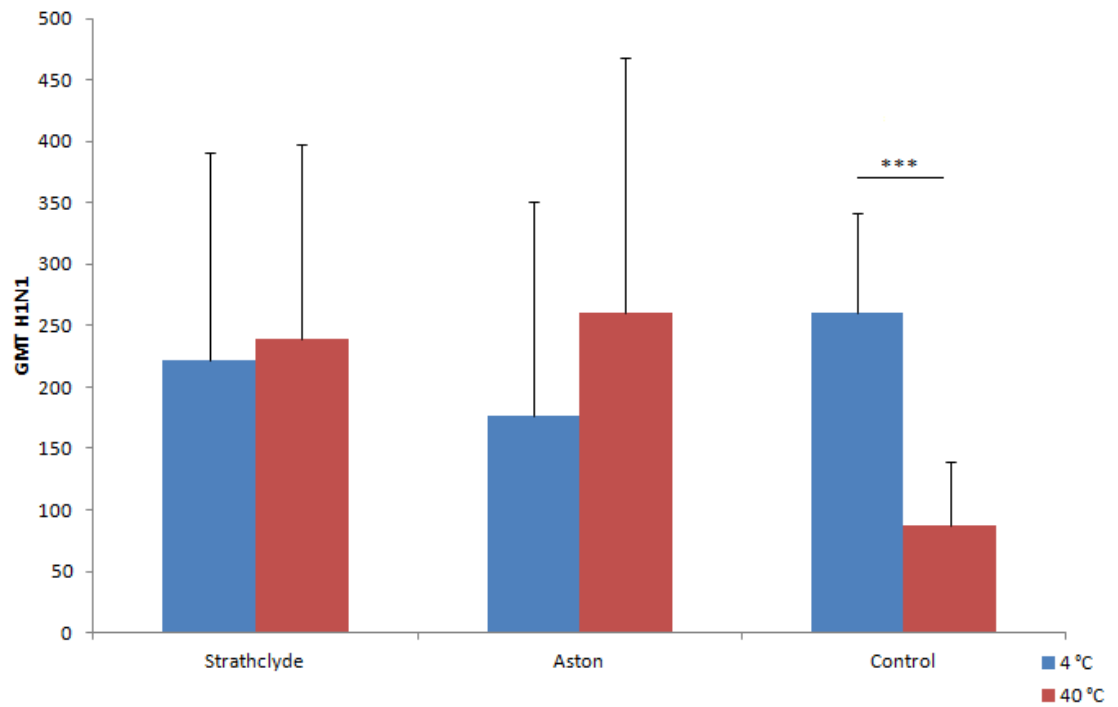
The use of delivery systems to provide immunogenicity has also been shown by Prego et al, (2010) using chitosan based microspheres to encapsulate hepatitis B antigen. Results from their studies show that the chitosan based microspheres are able to maintain antigen integrity (confirmed by SDS page) after lyophilisation and storage at 4 °C for a period of three months (Prego et al., 2010). Upon intramuscular administration of the Alum adsorbed hepatitis B antigen and the chitosan encapsulated hepatitis B antigen microspheres, there was a delay in the immune response for the microsphere vaccine compared to the alum vaccine (Prego et al., 2010). However, over time, significantly higher levels of IgG titres were produced with the chitosan microsphere based vaccine compared to the alum adsorbed vaccine, thus showing the effectiveness of encapsulating vaccine antigens with delivery systems to elicit immune responses.

4.3.6 Thermostability between the formulations

Given that the overall aim of this study was to develop potential thermostable vaccines, the next stage of this research was to consider the thermostability of the niosome formulations. Therefore, niosome vesicles incorporating the trivalent Fluzone 2009 antigens were prepared by the Aston melt and Strathclyde melt method and compared in terms of their ability to offer thermostability in a freeze-dried format. The niosome preparations outlined in table 4.1 were subjected to storage at 4 ± 3 °C and 40 ± 2 °C for 3 months and subsequently given as single intramuscular administration to Balb/c mice. Sera was collected prior to administration, as well as on day 14 and immunogenicity was assessed using the hemagglutination inhibition assay (HI assay) and direct ELISA. The commercial vaccine Fluzone was used as a positive control for comparison and stored in the same storage conditions as the niosome formulations.

The HAI assay is a serological technique which is used to detect HA-specific antibodies in serum resulting from infection or vaccination with influenza virus antigen; HAI titres correlate with protection from influenza in humans and a geometric mean titre (GMT) ≥ 40 is considered to be protective (Hobson et al., 1972, Hannoun et al., 2004). The HAI method works on the principle of the influenza viruses ability to agglutinate red blood cells with agglutination that is inhibited by anti-HA antibodies specific to the viral strain. The HAI antibody titres were expressed as the reciprocal of the highest serum dilution showing complete hemagglutination inhibition. With reference to the results of the preparations at 4 °C and 40 °C and the H1N1 strain (Figure 4.6 A), no significant difference in IgG titres were found between the mice immunised with either the Strathclyde melt and Aston melt niosome formulations and both formulation methods were comparable to the commercial vaccine titres.

A



B

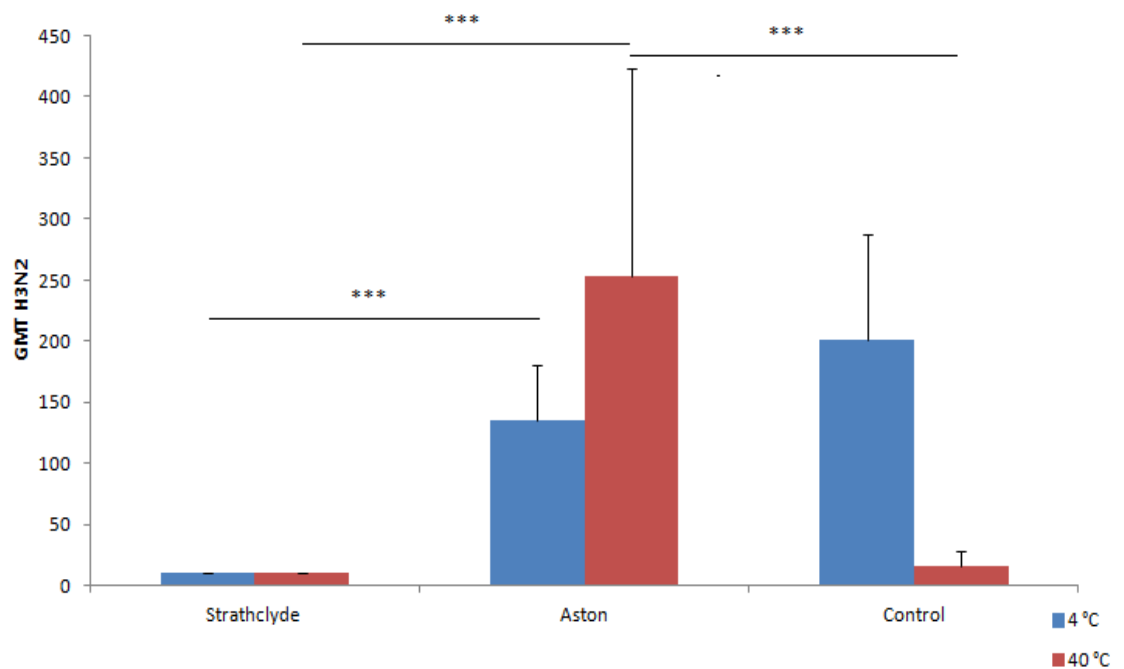


Figure 4.6: Geometric mean for IgG titres after administration of niosome vesicles at $t = 14$ days after formulations stored for 3 months at 4 °C and 40 °C for **A)** H1N1 and **B)** H3N2 ($n = 16$). ≤ 40 GMT considered protective against Influenza.

However, in terms of the preparations at 4 °C and 40 °C, responses against the H3N2 antigen (Figure 4.6B) were significantly different between all three immunisation groups. For the mice immunised with the Strathclyde melt method niosomes minimal (< 40 GMT) IgG titres were measured. In contrast, the Aston melt niosomes promoted highest IgG responses ($p < 0.01$) compared to both the control group and the mice which received the Strathclyde method niosomes, after storage at 40 °C, and when stored at 4 °C the IgG titres were comparable between the Aston melt and the commercial Fluzone control (Figure 4.6B). The control vaccine stored at 4 °C for both strains H1N1 and H3N2 showed significantly ($p < 0.01$) higher IgG titres than the control vaccine stored at 40 °C. These results suggest Aston melt niosomes can support antigen immunogenicity to be maintained after storage of the formulation at both 4 and 40 °C for a period of 3 months prior to administration.

One of the key changes in the preparation of niosomes implemented within the Aston melt method was that the process temperatures were reduced to between 30-35 °C. Both niosome preparations (Figure 4.1) were subjected to the same freeze drying protocol outlined in section 4.2.8. The freeze drying cycle exploited the lyoprotectant trehalose which is a natural alpha linked disaccharide which has known to protect proteins and stabilise lipid membranes during freeze drying (Crowe et al., 1987, Arakawa and Timasheff, 1982). Studies by Pizzuto et al, (2011) demonstrated that the incorporation of trehalose when freeze drying influenza antigens and subsequent storage at 37 °C and 45 °C for a period of two weeks to mimic failures in transport of antigens shows significant improvement in thermostability (Pizzuto et al., 2011). There were no significant differences observed in HA median titres of the antigens supplemented with trehalose when incubated at 37 °C and 45 °C therefore, improving the heat stability of the influenza antigens by maintaining their original HA titres. Therefore this would

suggest that the niosomes prepared by the Strathclyde melt were either initially less potent as vaccine delivery systems or were less stable during the freeze drying process. To confirm this, further studies comparing both niosome preparations pre and post formulation are required. However, given that the point of this work was to develop a thermostable vaccine protocol, the studies undertaken have demonstrated the niosomes prepared using the Aston protocol offer this attribute, at least 3 months. In addition, the lyophilisation process of liposome based vesicles with trehalose has been studied by Mohammed et al, (2007) where upon freeze drying of liposomes with trehalose has shown stability for a period of 42 days, showing retention of vesicle size and drug loading (Mohammed et al., 2007). Moreover, Gregoriadis et al, (1999) have demonstrated that material-containing liposomal suspensions can be freeze dried in the presence of a cryoprotectant (for storage) without significant loss of material from the vesicles on reconstitution (Gregoriadis et al., 1999). Within the study reported in Figure 4.5 the niosome formulation had been lyophilised and subsequently rehydrated prior administration and compared against a control formulation. Whilst no comparison to non-freeze-dried formulations were conducted, studies by Christensen et al, (2007) have previously confirmed that freeze drying of DDA/TDB vesicles when compared with a formulation prepared fresh prior to administration had no significant influences on antibody responses of the IgG1 and IgG2b isotypes (Christensen et al., 2007). Overall they found that freeze drying preserved adjuvant activity and that the antigen was not compromised where both a cell mediated and antibody response was produced. Therefore, as a result lyophilisation of sub-unit vaccine delivery systems are able to elicit immune responses greater than the antigen alone and are comparable to formulations prepared fresh.

Considering the differences between the strains shown in figure 4.6, studies by Coenen et al, (2006) on the stability of influenza sub-unit vaccines outside of refrigerator conditions conclude that the degradation rate of haemagglutinin differs between all the strains of influenza. For example, the A/Panama strain is susceptible to degradation 6-12 times faster when stored at 25 °C than at 5 °C (Coenen et al., 2006). Hence within this study, figure 4.6 shows that the H1N1 and H3N2 sub-unit antigens are both susceptible to storage conditions outside of the fridge (4 °C) where a significant reduction in IgG GMT titres is observed thus showing signs of haemagglutinin degradation when stored at 40 °C. The ability of the Aston melt method preparations to produce such high IgG titres when storing the vaccine at 40 °C for 3 months prior to administration has shown a major breakthrough in providing thermostability to influenza antigens when stored at elevated temperatures. The Aston melt niosomes showed no loss in potency of the two strains when stored at 40 °C and the results were comparable to the control vaccine stored at 4 °C.

4.4 Conclusions

With growing concern over the thermostability of vaccines globally, this chapter has focussed on the development of a vaccine delivery system that promotes thermal stability and dose-sparing. The advantage of using delivery systems is that the antigens can also be protected after administration (Gregoriadis et al., 1999, Gregoriadis, 1994). The revised method of preparing niosome vesicles (Aston melt) has shown to incorporate a larger quantity of antigen within the vesicle compared to current methods as has been demonstrated by the antigen protection levels. The key aspects of the revised method was a lowering of the process temperatures to 30-35 °C and the antigen being present prior to vesicle formation with the intention of protecting thermolabile antigens and incorporating more antigen within the vesicles. One of the common

problems resulting in vaccine wastage in several countries was due to power outages thus resulting in vaccines not being able to maintain their cold temperatures (Setia et al., 2002). The results within this study have shown that the niosome vaccine delivery system was able to protect the vaccine with no loss in potency for a period of 3 months at 40 °C, thus overcoming the problems associated with power outages and temperature increases worldwide.

The manufacture of thermostable niosomes, were shown to produce the most consistent immunological responses in animals for both H1N1 and H3N2 strains compared to the commercial Fluzone control vaccine. The results were confirmed by both HAI and ELISA techniques for formulations stored at 4 °C and 40 °C whereby the thermostable niosome vaccine (Aston melt method) showed its superiority in resulting in increased IgG titres. To note, the Strathclyde melt method did not show any immunological response against the H3N2 antigen when stored either at 4 °C or 40 °C suggesting that the antigen had been compromised. To conclude, this chapter demonstrates that there is great scope to use a wide range of antigens/drugs with this proposed method of producing thermostable niosomes that offer increased levels of protection and offer effective immunity.

Chapter 5

Trafficking and immunogenicity of orally administered bilosomes and niosomes

5.1 Introduction

5.1.1 Oral vaccination

The oral route is a potentially preferable route of delivery for vaccines due to its non-invasive nature, ease of administration and lack of infection risk from needle stick injuries. Most importantly, the mucosal immunity can be promoted by oral immunisation offering strong resistance against many pathogens that infect via the mucosal lining (Clark et al., 2001). The target site of such vaccines are the M cells, located in the Peyer's patches, which are responsible for promoting secretory IgA and other mucosal responses (Schmucker et al., 1996). However, even though the oral route is one of the most accessible routes and has good patient compliance, it is generally not feasible to administer vaccines via this route because of problems associated with low efficacy, due to the metabolism and degradation that occurs in the GIT. The gastrointestinal tract is lined with protease enzymes, such as trypsin and chymotrypsin, and this presents stability issues for orally administered proteins and peptides (Delie and Blanco-Prieto, 2005, Russell-Jones, 2000). In addition, the residence time of vaccines at the immune induction sites within the GIT (the Peyer's Patches) is limited due to GI transit. With this latter point, the size of the systems becomes important, as the vaccine needs to penetrate the intestinal epithelium with a short exposure time. Due to this problem, higher doses or an increased frequency of dosing are often required to supply sufficient antigen to elicit an immune response (Webster et al., 2003). However, it is essential to avoid dosing in quantities that could potentially lead to sustained low level secretion of antigen specific IgA, resulting from an increased systemic tolerance to the vaccine (Russell-Jones, 2000, Mowat, 2003).

To support the delivery of oral vaccines, bilosomes and niosomes have been considered to help offer protection in the biological milieu of the GIT and to protect the antigen.

However for such systems to promote immune responses both protection and appropriate uptake of the antigen is required. To facilitate this, niosomes formulated from Monopalmitoyl glycerol (MPG), Synthecol (Chol) and Dicapryl Phosphate (DCP) have been investigated (Mann et al., 2006). In addition, Sodium deoxycholate (EMD) has also been added to these formulations to further enhance their stability within the GIT. These so called bilosomes have been shown to stabilise vesicles against detrimental effects of bile acids in the GIT and have shown to provide adjuvant properties by inducing systemic and mucosal immunity when administered with antigens (Schubert et al., 1983, Mann et al., 2004) .

5.1.2 Aims and Objectives

Within this chapter the aim is to determine the effect of the method (Preparation temperature) of vesicle formulation, type of vesicle upon its *in vivo* trafficking and immunogenicity. This will be carried out by:

- Using a dual radio-labelling system where the antigen and the delivery system are tracked independently yet simultaneously *in vivo* and *in situ* when in the GIT.
- Determining the clearance of free antigen and the effects of niosomes versus bilosomes in the distribution of antigen and the delivery system.
- Looking at the effects of vesicle size and dosing strategy upon oral delivery of vaccines.

5.2 Materials and Methods

5.2.1 Surfactants

A range of non-ionic surfactant based bilayer vesicles were prepared by high shear homogenisation and analysed using several materials and methods. The surfactants used

in the study were Monopalmitoyl glycerol (MPG) (Larodan Labs, Sweden), Synthecol (Chol) (Sigma Aldrich, UK), Dicetyl Phosphate (DCP) (Sigma Aldrich, UK) and Sodium Deoxycholate (EMD, UK). The aqueous buffer sodium bicarbonate (Sigma Aldrich, UK) was buffered to pH 7.6 using a 1 M sodium hydroxide solution (Sigma Aldrich, UK).

5.2.2 Radio-labelling of H3N2 antigen

The fluzone vaccine is commercially available in PBS and not as a concentrate and comprises of additives such as gelatine which interfere in centrifugation and labelling (Table 5.1). The fluzone is made up of three recombinant HA antigens which are mixed together to form the vaccine. As a result three individual rHA strains were obtained which could be used be radio-labelled with ^{125}I using a previously described method (Henriksen-Lacey et al., 2010a) so to facilitate the quantification and tracking of the antigens. The three antigens included within fluzone are: H1N1 (75 kD), H3N2 (75 kD) and Brisbane (100 kD) and each of the individual antigens were labelled with ^{125}I .

Table 5.1: Constituents forming Fluzone vaccine

Component	Quantity per dose
Active substance (Inactivated strains)	45µg HA total
H1N1	15 µg HA
H3N2	15 µg HA
Brisbane	15 µg HA
Sodium phosphate buffered isotonic solution	To appropriate volume
Formaldehyde	≤100 µg
Octylphenol ethoxylate	≤100 µg
Gelatine	0.05%

Dual radiolabelling techniques have been used widely within our lab to follow the movement of vaccine antigens and their delivery systems (Henriksen-Lacey et al., 2010a). It enables the antigen to be tracked using one radioisotope and at the same time to track the antigen carrier system using a different radioisotope. Dual-radiolabelling increases the data output of the study whilst keeping in line with the 3 Rs of reduce, refine and replace of animal models.

The H3N2 antigen (Immune Tech, USA) stock solutions were prepared as 100 µg of purified protein (1 mg/mL) in PBS stock and 40 µL (40 µg) placed into an iodination tube (Pierce Biotechnology) with 2 Mbq of ¹²⁵I (Perkin Elmer, Belgium) for 1 hour. A separation column was pre-prepared by firstly, heating Sephadex G75 (Sigma Aldrich, UK) beads for 3 hours at 90 °C and left to cool, during that time the column was prepared by placing glass wool at the bottom to prevent any Sephadex beads from falling through the column. The column was then packed using the pre heated and swollen Sephadex G75 prepared earlier. It is important that the gel remains hydrated with the PBS buffer which is used for labelling. It is essential to optimise the flow rate based on sample collection and then to match the sample volume collected to the correct protocol on the gamma counter.

Upon packing of the column, just above gel, the solution from the iodination tube was added to the top of the column and then rehydrated with buffer and samples were collected every 1 minute for 1 hour which results in the collection of 500 µL per vial. The vials were then analysed for ¹²⁵I content using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA) individually to prevent any backscatter that may occur from neighbouring vials. To confirm presence of protein in the vials a micro- BCA assay was carried out and used in parallel with the data obtained from the iodination of the protein.

5.2.3 Preparation methods of vesicles

5.2.3.1 Preparation of niosomes using Aston melt method

A 5:4:1 molar ratio of MPG, Chol and DCP was weighed out and placed into a 10 mL glass beaker. Before melting the lipids the aqueous phase was prepared in a 15 mL falcon tube. 25 mM sodium bicarbonate (pH 7.6) formed the bulk part of the aqueous phase and was spiked with radiolabelled antigen (Section 5.2.2) and then placed in a heated water bath for 10 minutes at 30-35 °C. This was to be known as the buffered antigen stock solution.

While the aqueous buffer was preheated, the beaker containing the lipids was placed into a hot oil bath (120-125 °C) and melted for 10 minutes. The beaker containing the molten mixture was removed from the oil bath and the buffered antigen stock solution was immediately added, the mixture homogenised at 8000 rpm at 30-35 °C for 10 minutes, and left to incubate at 220 rpm at 30 °C for 2 hours.

5.2.3.2 Preparation of bilosomes using Aston melt method

A 5:4:1 molar ratio of MPG, Chol and DCP was weighed out and placed into a 10 mL glass beaker. Before melting the lipids, the aqueous phase was prepared in a 15 mL falcon tube. 25 mM sodium bicarbonate (pH 7.6) formed the bulk part of the aqueous phase containing 100 mM sodium deoxycholate (bile salt). Radiolabelled antigen (Section 5.2.2) was then placed in a heated water bath for 10 minutes at 30-35 °C. While the aqueous buffer was preheated, the beaker containing the lipids was placed into a hot oil bath (120-125 °C) and melted for 10 minutes. The beaker containing the molten mixture was removed from the oil bath and the buffered antigen stock solution is immediately added to this beaker and then immediately homogenised at 8000 rpm at

30-35 °C. The mixture was homogenised for 10 minutes and is then left to shake and incubate at 220 rpm at 30 °C for 2 hours.

5.2.3.3 Preparation of bilosomes using Strathclyde melt method

A 5:4:1 molar ratio of MPG, Chol and DCP was weighed out and placed into a 10 mL glass beaker. 25 mM sodium bicarbonate (pH 7.6) formed the bulk part of the aqueous phase. 100 mM bile salt was prepared in 25 mM sodium bicarbonate buffer. The two buffered solutions were placed into a heated water bath at 60 °C. A 100 µL radiolabelled antigen was then placed in a heated incubator for 5 minutes at 37 °C. While the aqueous buffer was heated, the beaker containing the lipids was placed into a hot oil bath (120-125 °C) and melted for 10 minutes. The beaker containing the molten mixture was removed from the oil bath and the 25 mM sodium bicarbonate buffer added to this beaker and then immediately homogenised at 8000 rpm at 60 °C for 2 minutes. Upon two minutes homogenisation, 0.55 mL of the 100 mM bile salt solution was added and further homogenised for three minutes. Upon 5 minutes total homogenisation time the radiolabelled antigen (Section 5.2.2) was added to the homogenising suspension and homogenised for a further five minutes and then left to shake and incubate at 220 rpm at 30 °C for 2 hours.

5.2.4 Characterisation of vesicles

The size of the vesicles was determined using laser diffraction on a sympatec 2005 (Helos/BF) analyser. 20 µL aliquots of the vesicle suspension was diluted into the cuvette with 40 mL double distilled water. The zeta potential, which is an indirect measurement of the vesicle surface charge, was measured in 1.5 mL double distilled water at 25 °C on a Zeta Plus Brookhaven Instrument. 20 µL of the bilosome suspension was mixed in 1.5 mL double distilled water and then analysed. The pH of

the vesicle suspension was determined using a pH meter where the tip was placed into the vesicle suspension and left for a few minutes.

5.2.5 Quantification of antigen

For quantification of antigen association, ultra-centrifugation of the formulations was required to isolate antigen entrapped vesicles, from non-incorporated antigen. To achieve this, 300 μ L aliquots of sample was diluted in a Beckman 3.9 mL Polo-allomer tube and filled up to the neck with double distilled water. The tubes were heat sealed ensuring that there are no air bubbles present within the tube. The tubes were then placed accordingly in the TLN-100 rotor and centrifuged at 354,000 X g for 45 minutes at 4 °C. When centrifugation was complete, the tubes were opened and the supernatant removed. The sample was resuspended and the centrifugation step repeated. After the second ‘wash’ the antigen loaded vesicles are then re-suspended with 100-200 μ L of appropriate buffer and transferred all to a clean/labelled 1.5 mL Eppendorf tube.

5.2.6 Stability of the vesicles in simulated fasted gastric and intestinal medium

Fasted state simulated gastric media (50 mL) was prepared using a 34.2 mM NaCl solution in 50 mL HPLC water at pH 1.2 adjusted with 1 M HCl. Pepsin (40 mg) was then added, followed by sodium taurocholate (2.15 mg) and phosphatidylcholine (0.76 mg) in 50 mL at 37 °C (Vertzoni et al., 2005).

Fasted state simulated intestinal media (50 mL) was prepared by a 50 mM PBS solution in 50 mL HPLC water at pH 8.5, adjusted with 1 M NaOH, and sodium glycodeoxycholate (180 mg) and phosphatidylcholine (34 mg) dissolved in the solution at 37 °C. To establish the effect of these conditions on vesicle attributes, 400 μ L of the vesicle formulations was added to 3.6 mL of fasted gastric medium as a 1:10 dilution. The formulations were tested for vesicle size and zeta potential at specific time

intervals. The gastric to intestinal phase was carried out by centrifuging 3.9 mL of the formulations from the gastric period and then resuspending the pellet in fasted intestinal fluid and was then tested at the stated time points. To measure antigen retention in these conditions, radio-labelled antigen was incorporated within the formulations and antigen retention tracked in the above conditions by ultracentrifugation (Beckman, Ultima XP) at 354,000 X g.

5.2.7 *In vivo* biodistribution protocol

Inbred female Balb/c (6-10 weeks of age) mice were housed in cages within a laminar flow safety enclosure and provided with irradiated food and filtered drinking water. Experimentation adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subject to ethical review and were carried out in a designated establishment. 200 µL doses of the formulations, which were washed to remove untrapped radiolabelled antigen, were given orally to Balb/c mice in groups of 4. Animals were terminated at various time points, organs collected and analysed for both ^{125}I (to measure antigen) and ^3H , which was used as a radio-active tracker for vesicles by incorporating ^3H -cholesterol in the formulation (Figure 5.1).

Gamma vials were pre-labelled and individual tissues/organs were weighed and individually placed into the gamma vials. To the gamma vials 1.5 mL of solvable (Perkin Elmer, UK) was added to digest the tissues. Once the solvable was added to the vials they were then placed onto the gamma counter to record the ^{125}I -antigen levels. The vials were then placed into an incubator at 50 °C overnight to dissolve the tissues. Once the tissues had dissolved the contents of the gamma vials were transferred to 20 mL scintillation vials where 200 µL hydrogen peroxide (Sigma Aldrich, UK) was added to each vial to bleach the samples. The vials were left overnight once again to wait for

gas to disappear and 10 mL of Ultima Gold scintillation fluid (Perkin Elmer, UK) was added to form an emulsion. The vials were then counted on the scintillation counter which will represent the counts for the vesicles at each site.

5.2.8 Calibration of Radioisotope equipment

The presence of ^{125}I in scintillation vials results in interference within the scintillation counter when counting for tritium; hence, a calibration was carried out where known iodine concentration counts were serially diluted and counted on both counters to confirm the readings. The calibration curves were merged together and an equation formed hence allowing recalculation of the final counts (Figure 5.2).

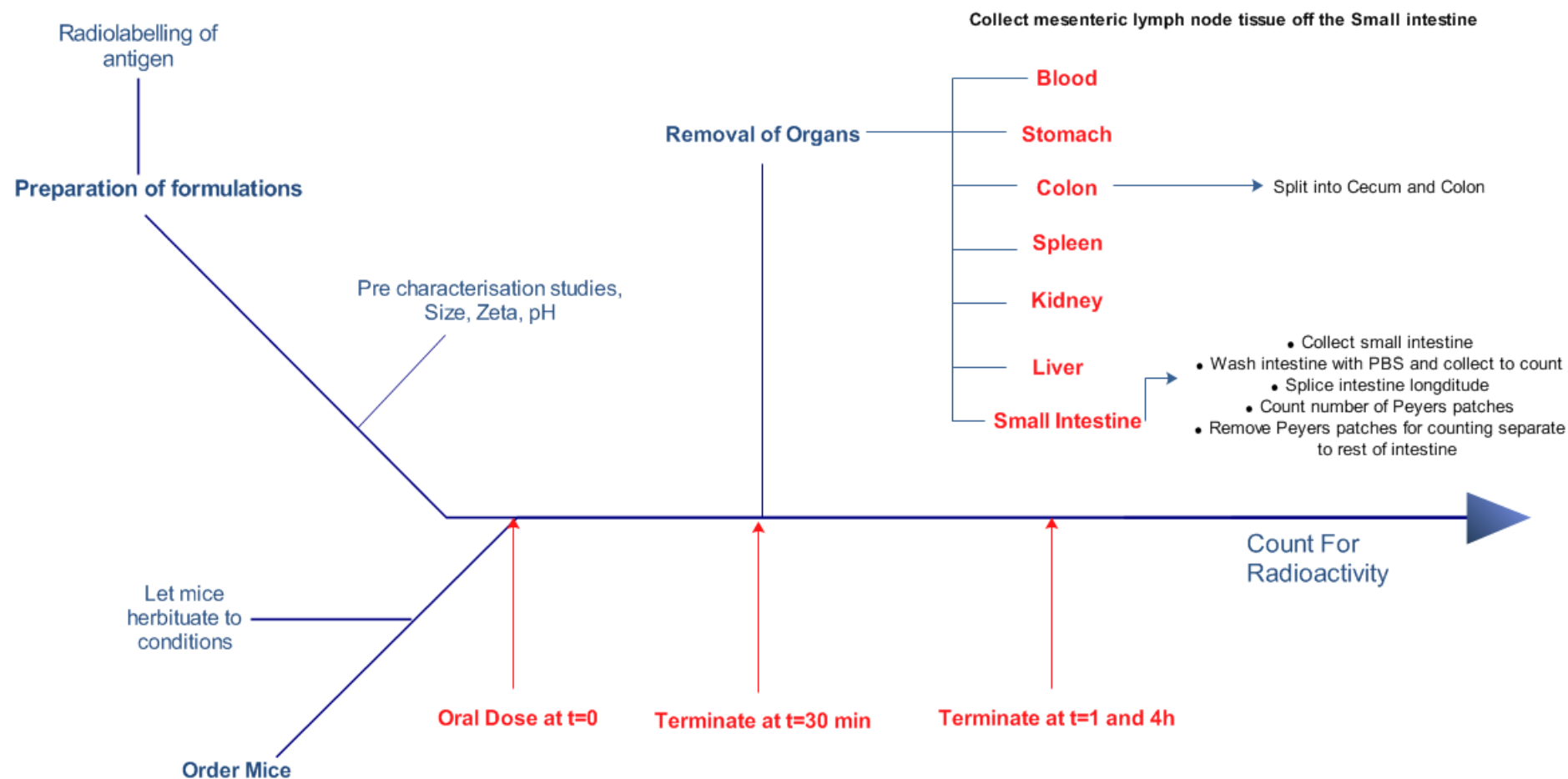
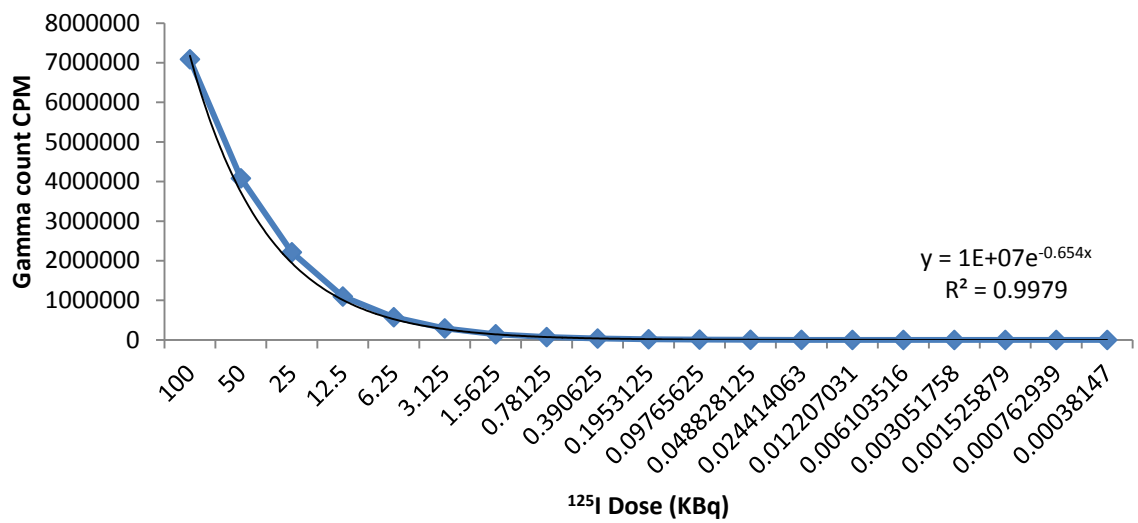


Figure 5.1: Biodistribution regime showing plan of termination and extraction of GI organs at the various time points.

A



B

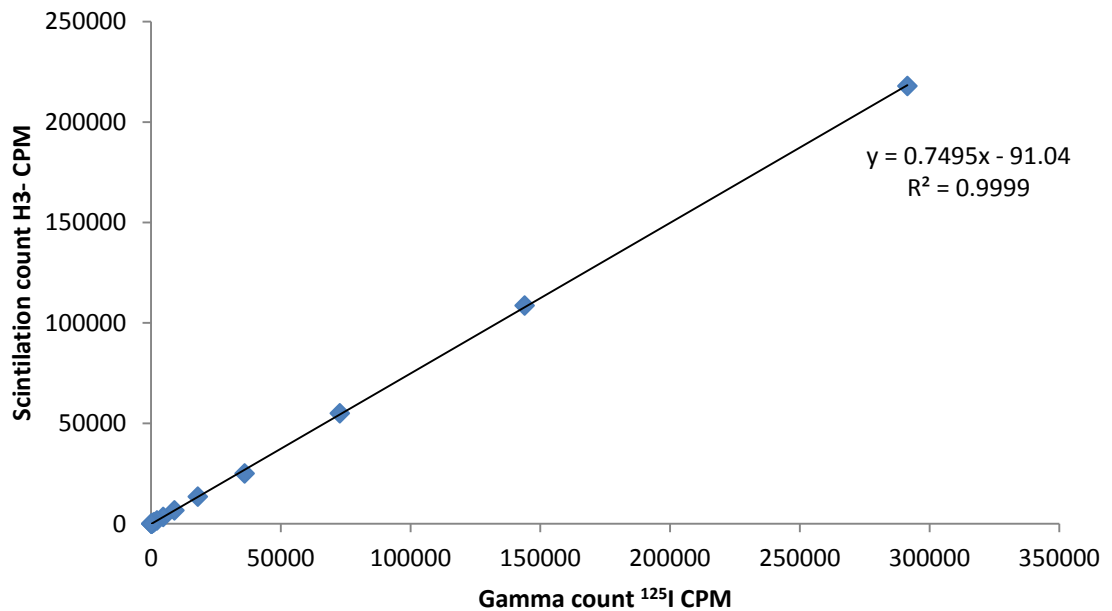


Figure 5.2: Calibration of Radioactivity counters: **A)** serial dilution of ^{125}I dose for calibration on gamma counter and **B)** Calibration curves for ^{125}I against interference on scintillation counters (n=3).

5.2.9 Statistical analysis

The results within this study are given as the geometric mean \pm S.D. unless stated otherwise. The statistics were carried out using ANOVA and a probability factor of less than 0.05 ($p < 0.05$) was considered to represent statistically significant difference.

5.3 Results and Discussion

5.3.1 Physical characteristics of vesicles when subjected to fasted-state GI fluids

Non-ionic surfactant based vaccine vesicles have provided effective immunity in various animal models such as mice and ferrets after oral administration (Brewer and Alexander, 1992). The oral route provides a challenging environment for delivery of antigens including low pH, digestive/ gastric enzymes the poor absorption and the rapid transit (Singh and O'Hagan, 1998). As a result, it is important to ensure that the antigen and carrier system remains intact during its transit through the GI tract until it reaches the target site. Therefore, vesicles were subjected to simulated gastric (pH 1.2) and intestinal media (pH 8.6) and at various time points the vesicles were analysed for vesicle size and zeta potential.

The formulations of niosome and bilosomes were analysed for vesicle size (Figure 5.3A) and zeta potential (Figure 5.3 B) when placed into 1) fasted gastric media, 2) fasted gastric to intestinal fluid (GSIF), and 3) fasted intestinal fluid (SIF). Results (Figure 5.3A) demonstrate that niosomes formulated by the Aston melt method show a significant ($p < 0.05$) decrease in overall VMD from $6.54 \pm 0.04 \mu\text{m}$ at $t=0$ to 5.46 ± 0.05 (after 1 h gastric media) to $3.57 \pm 0.03 \mu\text{m}$ after a period of 4 hours in the GSIF medium (Figure 5.4 A). In contrast, the Aston bilosome vesicles significantly increase in VMD ($p < 0.05$) from $6.19 \pm 0.04 \mu\text{m}$ ($t=0$) when in gastric medium to $9.13 \pm 0.31 \mu\text{m}$ ($t=1 \text{ h}$) and then return to their original vesicle size when in GSIF to $6.11 \pm 1.27 \mu\text{m}$ ($t= 4 \text{ h}$) which could be due to flocculation of vesicles within the gastric medium. In comparison, the Strathclyde bilosomes vesicles follow a similar trend as the Aston bilosomes vesicles. However, the extent of the increase in vesicle size is significantly larger ($p < 0.05$) in the gastric medium of a VMD of $11.9 \pm 0.57 \mu\text{m}$ in relation to the Aston bilosomes of $9.13 \pm 0.31 \mu\text{m}$.

In addition, the study of zeta potential surface charge (Figure 5.3B) was carried out of the vesicles in the various media. A sample of the formulation was taken at each timepoint and suspended into a 100 fold diluted medium of gastric or intestinal fluid depending upon which sample was being analysed. Results show that when in the stomach the zeta potential at pH 1.2 results in a significant decrease ($p < 0.05$) in surface charge around -35 mV and when in the duodenum region of pH 6 surface charge significantly ($p < 0.05$) returned to -80 mV (as represented by the pH titration graph in Chapter 2) and then finally when at the site of uptake where the pH in the fasted state is 8 the zeta potential returns to its original value of -100 mV. This trend is observed for all three formulations and is represented in figure 5.3B. Hence, the drop in zeta potential within gastric conditions for all formulations could potentially encourage flocculation, however, the niosome vesicles showed no increase in vesicle size. Thus, suggesting that the bilosome preparations which increase in vesicle size, reduce in zeta potential when in gastric media flocculate as this trend is reversible when in intestinal media suggesting that the bile salt component could be promoting flocculation.

It is important that the vesicle size and surface charge remain stable and are fit for purpose as studies have shown that the proposed transparacellular uptake of particles within the Peyer's patches is restricted to particulates below 10 μm (O'Hagan, 1996, Eldridge et al., 1990, van der Lubben et al., 2001). Furthermore, the retention within the Peyer's patches has been demonstrated for vesicles between 3 -10 μm , whilst vesicles below 3 μm translocate into the lymphoid tissue (Eldridge et al., 1990, Ebel, 1990). In addition, a study using microspheres by Tabata et al, (1996) have shown that particles within the 5-10 μm induced a mucosal immune responses whereas smaller vesicles migrated into the lymphatics resulting in systemic immunity (Tabata et al., 1996). As a result this study proves that the vesicles prepared for the delivery of antigens fall within

an acceptable range for uptake and retention within the Peyer's patches so when incorporated with antigen will induce a mucosal immune response. Although the vesicles vary in size and charge upon exposure to the milieu within the GIT they do however, fall within acceptable ranges for uptake by the Peyer's patches. In addition to the vesicle size and charge, a key parameter is whether the antigen remains associated to the vesicles throughout the transit within the GIT and to determine how much is prematurely released or lost before reaching the target site.

5.3.2 Antigen retention of vesicles when subjected to fasted GI fluids

For therapeutic application, it is essential that the antigen is retained within the delivery system to enhance targeting. Given that GI conditions were shown to impact on vesicle size and zeta potential, this is an important factor as this could influence antigen retention whether the antigen is entrapped or surface adsorbed to the vesicles. Therefore to investigate the ability of the vesicles to retain antigen during GI transit, vesicles containing antigen were subjected to a full simulation of GI conditions as previously conducted (section 5.3.1) but for the determination of antigen release. This is critical for the oral route, as the surface of the vesicles is exposed to the internal milieu of the GIT and is exposed to changes in pH, digestion enzymes and mucous proteins.

H3N2 antigen was radiolabelled and incorporated into the niosome and bilosome vesicles (see section 5.2.3) and antigen retention studies were carried out (section 5.2.5) where the vesicles were centrifuged to determine total antigen association. Entrapment shows that initial antigen loading was significantly higher ($p < 0.01$) in niosomes compared to bilosomes ($39.21 \pm 2.72\%$ vs $32.5 \pm 2.9\%$; Figure 5.4 respectively). Results show that just after 15 minutes in gastric media the niosome preparation significantly decreases ($p < 0.05$) in antigen retention to $28.04 \pm 0.95\%$ whilst the bilosome preparations maintain antigen retention with no significant loss.

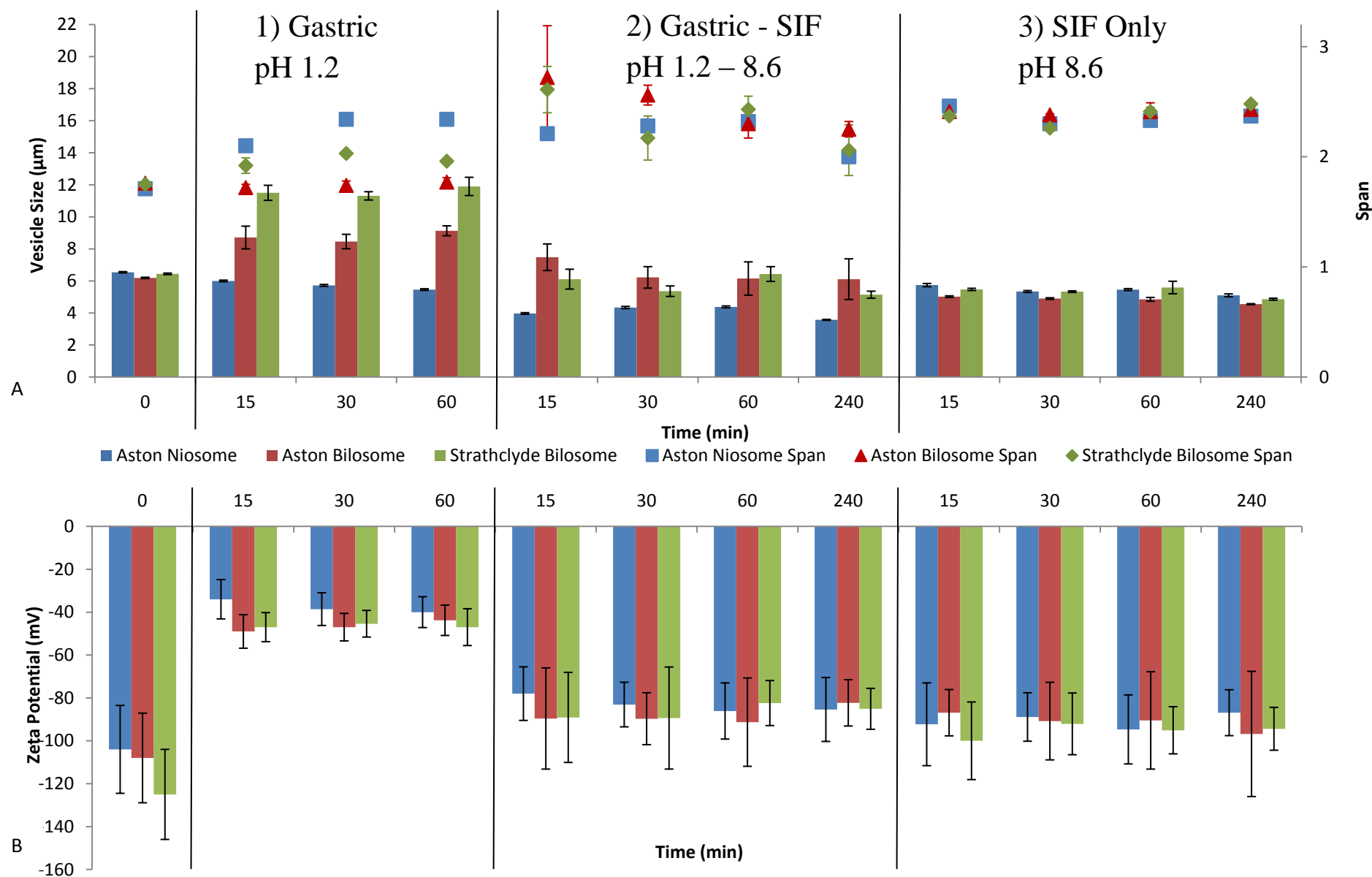


Figure 5.3: A) VMD size distribution for vesicles subjected to GI fluids at various time points, **B)** zeta potential readings for the formulations in part A. Blue bars represent Aston niosomes, red bars represent Aston bilosome and the green bars represent Strathclyde bilosomes (n=3).

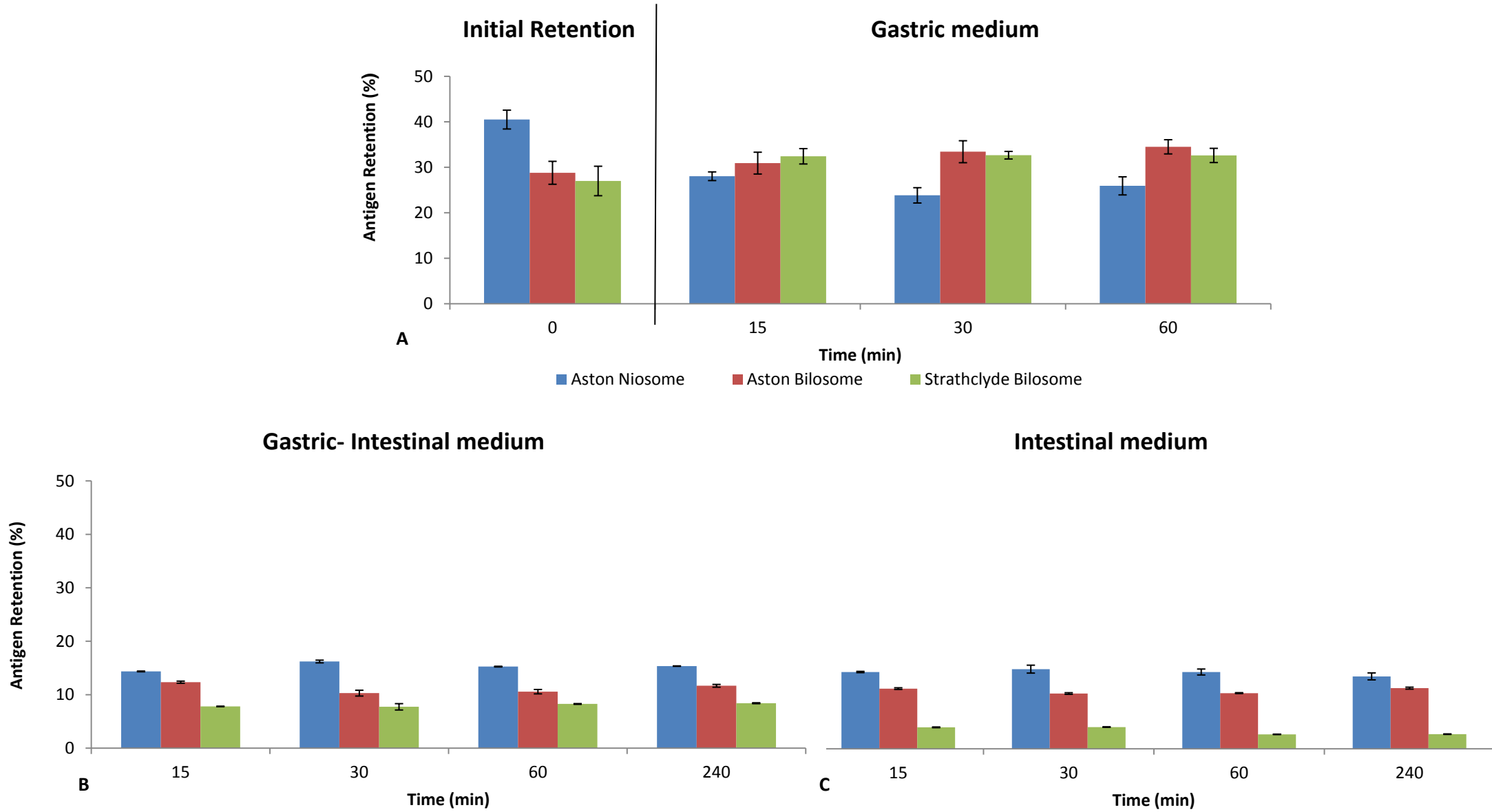


Figure 5.4: Antigen retention within vesicles at various time points corresponding to the formulations in figure 5.3 when subjected to **A)** Gastric media, **B)** Gastric – Intestinal media and **C)** Intestinal media.

An in depth look into the antigen retention within the vesicles for the three formulations showed that overall, antigen retention decreased in all formulations with the greatest reduction in the Strathclyde bilosome formulation and Aston niosome formulation (Figure 5.4). Figure 5.4A shows that the niosome preparation has lost antigen retention whilst the two bilosome preparations maintain their loaded antigen at all times within the gastric media. However, upon subjecting the vesicles to GSIF from the gastric media (Figure 5.4B), antigen retention significantly decreases ($p < 0.05$) for all formulations. Antigen retention when in SIF media is greatest within the niosome formulation which could be due to the initial higher antigen association than that of the two bilosome formulations (Figure 5.4C). The sharp decline in antigen retention with the Strathclyde bilosome formulation (Figure 5.4C) when placed into the SIF of 2-4 % compared to the Aston bilosome significantly higher recovery ($p < 0.05$) of 12 % at the same conditions could be an indication of where the antigen resides within the vesicles; due to their differences in preparation the antigen in the Strathclyde melt preparation could be surface adsorbed to a greater extent than that of the Aston melt preparations. Due to this surface adsorption, the intestinal medium could be degrading the antigen located on the surface or outer regions of the vesicles hence resulting in 2-4 % antigen retention when in the fasted intestinal fluid. Kaur et al, (2012) also demonstrated that antigen surface adsorbed to DDA:TDB liposome vesicles were only able to retain 20.8 ± 7.8 % antigen after trypsin digestion compared to vesicles prepared by a dehydration-rehydration method; where antigen was able to entrap itself within the bilayers thus offering protection from enzyme digestion with 66 ± 1.2 % antigen retained (Kaur et al., 2012). Hence, location of antigen within bilayer vesicles are able to dictate antigen retention levels where antigen which is not surface bound is potentially protected from enzymatic degradation.

This suggests that the antigen content is consistent within the aqueous core of the vesicles as when formulating the vesicles via the Aston melt method where, the antigen is in the aqueous phase pre homogenisation hence having a greater chance to be incorporated within the aqueous core in comparison to the Strathclyde method where the antigen is added at a later stage during the homogenisation procedure. Thus, the incorporation of antigen within the buffer used to form the vesicles and at the revised lower manufacturing temperature (30 °C) is a good opportunity to increase antigen concentration at the target site and to allow the use of thermolabile antigens within this delivery system.

The use of carrier delivery systems have been shown in several studies to protect drugs/ antigens thus aiding in stability, biodistribution, drug transport and release via the oral route (Mane and Muro, 2012, Torchilin, 2009, des Rieux et al., 2006). Within this study, it has been shown that the redesigned niosome and bilosome method offers increased antigen protection *in vitro* than the previous published bilosome method. Mane and Muro, (2012) also demonstrate that degradation of IgG *in vitro* at various pH conditions is significantly higher than when incorporating the IgG with nano-carriers (Mane and Muro, 2012). Furthermore, Shukla et al, (2011) demonstrate that bilosome vesicles entrapping diphtheria toxoid offer protection through the GIT and increased uptake by the M cells (shown by confocal microscopy) compared to the free dose (Shukla et al., 2011). These simulated GI conditions within this study have shown that the vesicles are able to withstand the harsh environment and that antigen can be protected and retained within the vesicles from exposure to the external milieu of the GIT, thus offering increased antigen bioavailability when reaching the target site. As a result, the vesicles and antigen were fluorescently labelled to track the vesicles within the GIT and to determine whether they reach the target site intact.

5.3.3 Confocal laser scanning microscopy of vesicles

Within this study we have shown that the vesicles remain within the desired size range 5-10 μm after subjecting to simulated gastric and intestinal conditions. To obtain a greater understanding of antigen location within the vesicles, confocal laser light microscopy of the Aston niosome vesicles was carried out. The bilayers of the vesicles were fluorescently labelled with a lipid dye- Dil-C and the antigen was fluorescently labelled with a fluorophore dye. Figure 5.5 represents the imaging process from manufacturing of the vesicles to the site of uptake of the vesicles/ antigen *in vivo*.

As represented within figure 5.5, the bulk labelled antigen (labelled blue with Flammaflour) resides within the aqueous core of the Aston niosome vesicles as distinguished between the red bilayers and the blue core in figure 5.5 compared to the niosomes without loaded antigen which shows an empty clear aqueous core (Figure 5.5). Due to the limitations of the microscopy technique the high laser intensity results in rapid photo-bleaching of the antigen fluorescence so attempts to zoom into the bilayer regions and the surface of the vesicles to locate any surface bound antigen proved to be difficult.

The fluorescently labelled vesicles were administered via oral gavage (0.2 mL dose) and after 30 minutes tissues were excised and analysed via microscopy to locate the vesicles. Figure 5.5 shows the procedure start to finish in terms of how the vesicles appear under confocal microscopy after centrifugation and once they have been administered orally. The figure shows that upon excising of the Peyer's patch the vesicles are intact, spherical and between 5-10 μm .



The vesicles can be observed in large numbers when on a low magnification and upon increased magnification and 40 μm deep within the tissue a 5 μm vesicle is clearly visible hence suggesting that the Peyer's patches take up the vesicles. Moving from the Peyer's patches to the underlying mesenteric lymph tissue once again fluorescence is visible with a green autofluorescence (from collagen within the capillary network) within the mesentery. As a result, this study shows that vesicles are able to penetrate and reside within the Peyer's patches.

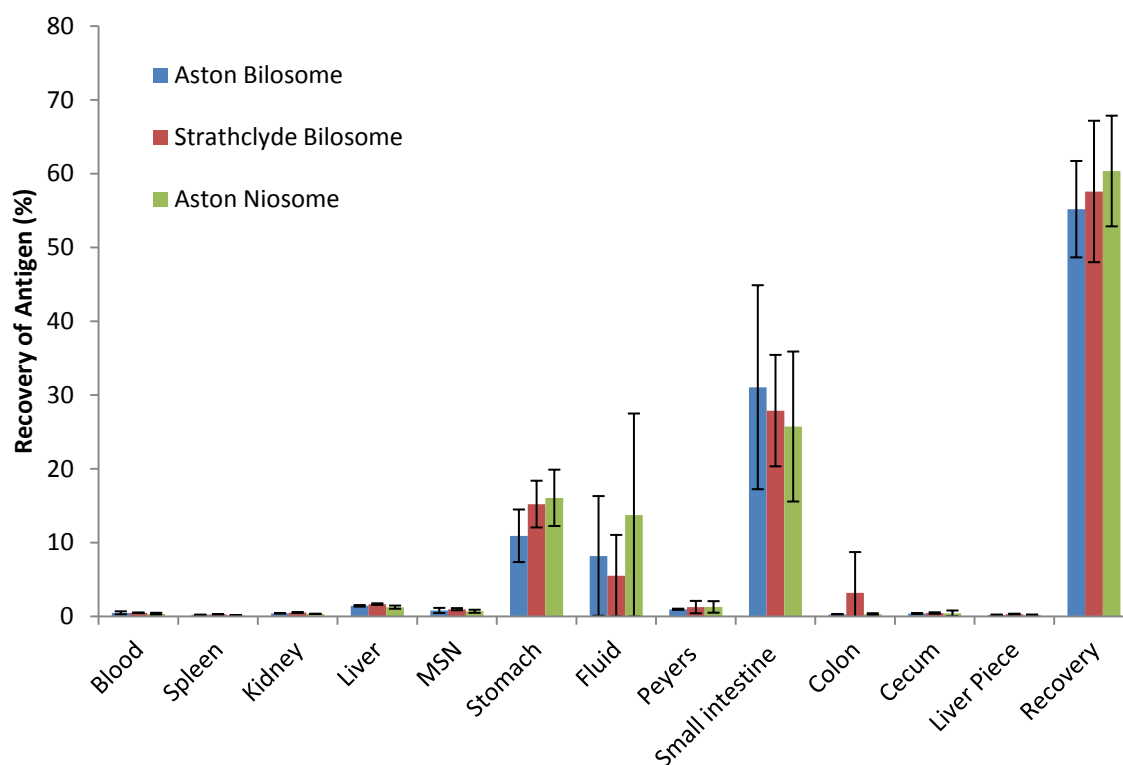
In terms of clearly distinguishing between the vesicle and the antigen this was a challenge as the limitations of the confocal result in a multiphoton laser being emitted which shows excitation at all wavelengths and in doing so the antigen and vesicle are not distinguishable. Furthermore, it is also undistinguishable whether the fluorescence is from clusters of vesicles or whether the vesicles are destroyed and distorted. Therefore to further investigate *in vivo* trafficking of the vesicles and antigen a dual radiolabelling technique was used to determine the antigen/vesicle retention at sites across the GIT.

5.3.4 Biodistribution of mucosal vaccines

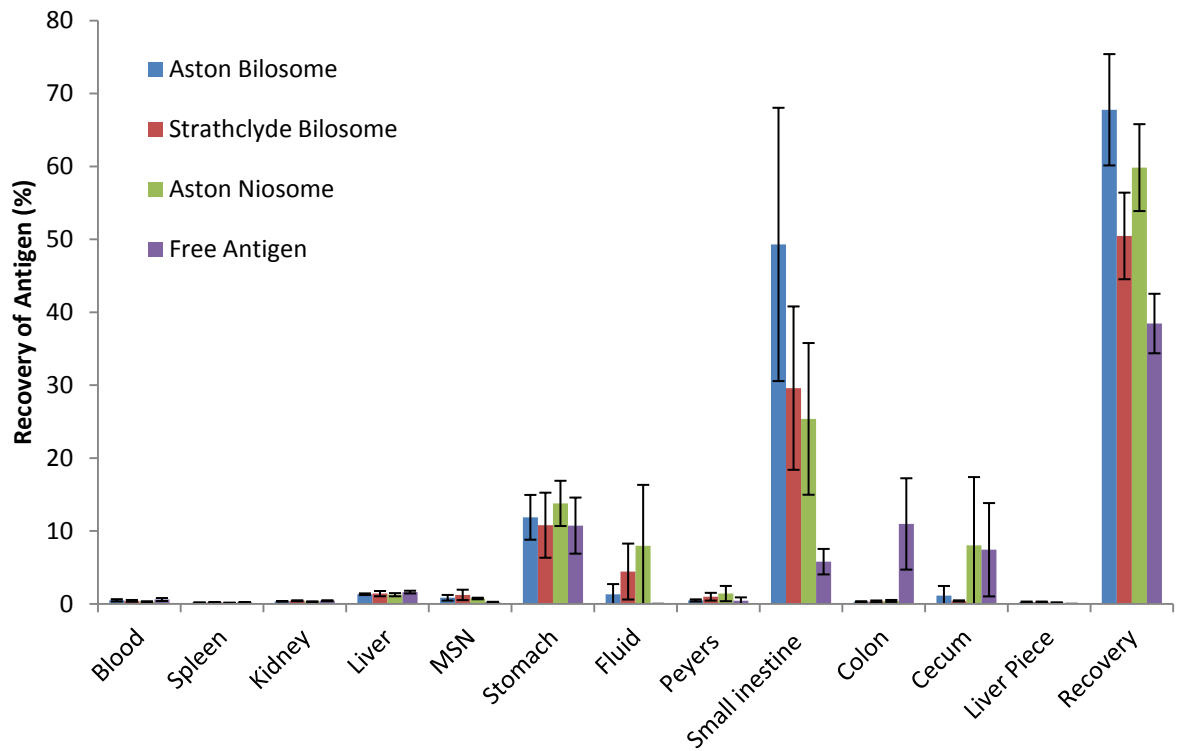
It is important to understand the biodistribution of mucosal vaccines administered orally as they are subject to dilution in mucosal secretions, capture within the mucus and attack by the mucosal proteases and enzymes with the largest obstacle of exclusion by the epithelial barriers (Neutra and Kozlowski, 2006). Within this study, the location and biodistribution of H3N2 antigen (^{125}I) and the vesicle carrier system (^3H - Chol) was used to understand the uptake of vesicle and antigen and to determine the residence time of the vaccine within the GIT. An oral gavage volume of 0.2 mL was used based on an upper limit of oral dosing of 20 mL Kg^{-1} (McConnell et al., 2008, Wolfensohn and Lloyd, 2003).

The results from the study are presented in Figure 5.6, where the ^{125}I counts represent the tracking and movement of the H3N2 antigen and the ^3H -chol represents the tracking and movement of the vesicle. Figure 5.6 represents the Aston bilosomes, Aston niosomes and Strathclyde bilosomes formulations at time intervals of 30 minutes, 1 hour and 4 hour termination of the test subjects. The 1 hour time point also includes the free antigen which was administered in the same way as the formulation by oral gavage. The general trend observed from figure 5.6 shows that the blood, spleen, kidneys and liver have trace amounts of antigen and vesicle recovery with the majority of the recovery coming from the stomach, small intestine, and the colon and cecum, suggesting minimal systemic absorption of particles.

A



B



C

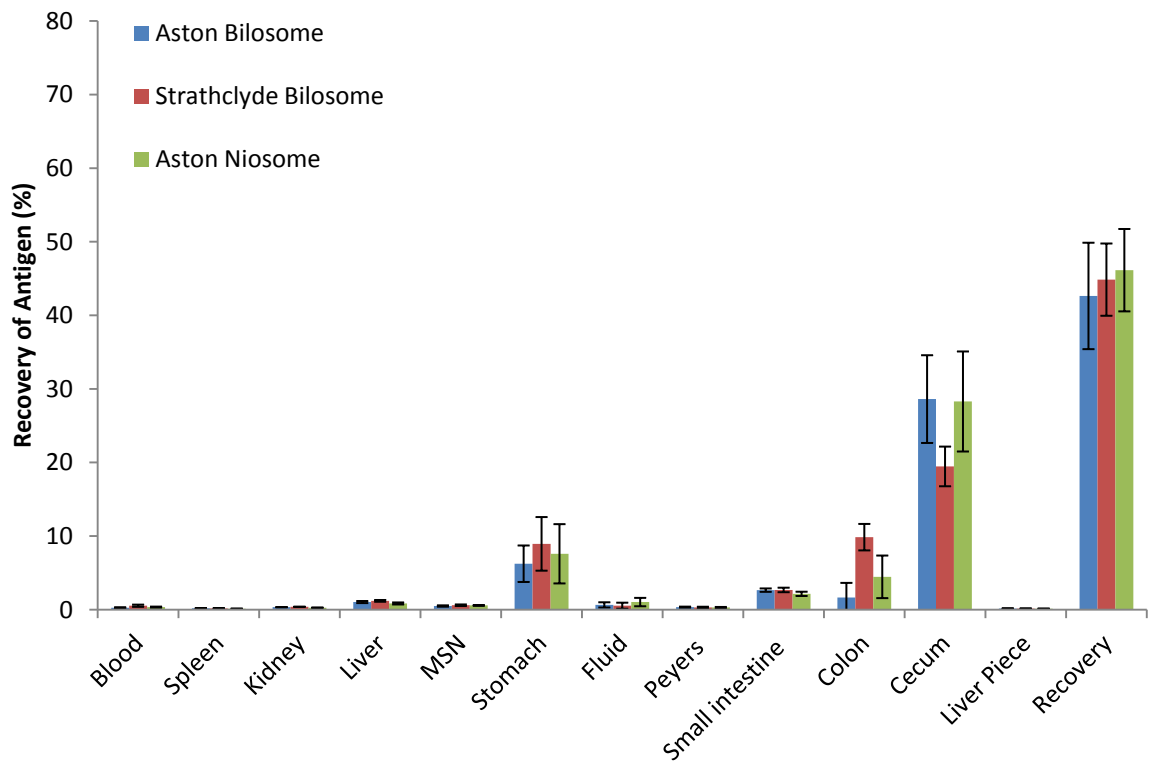


Figure 5.6: Antigen recovery after dissection and removal of organs based on initial radioactive dose counts , **A)** time point 30 minutes, **B)** time point 1 hour and **C)** time point 4 hours (n=4).

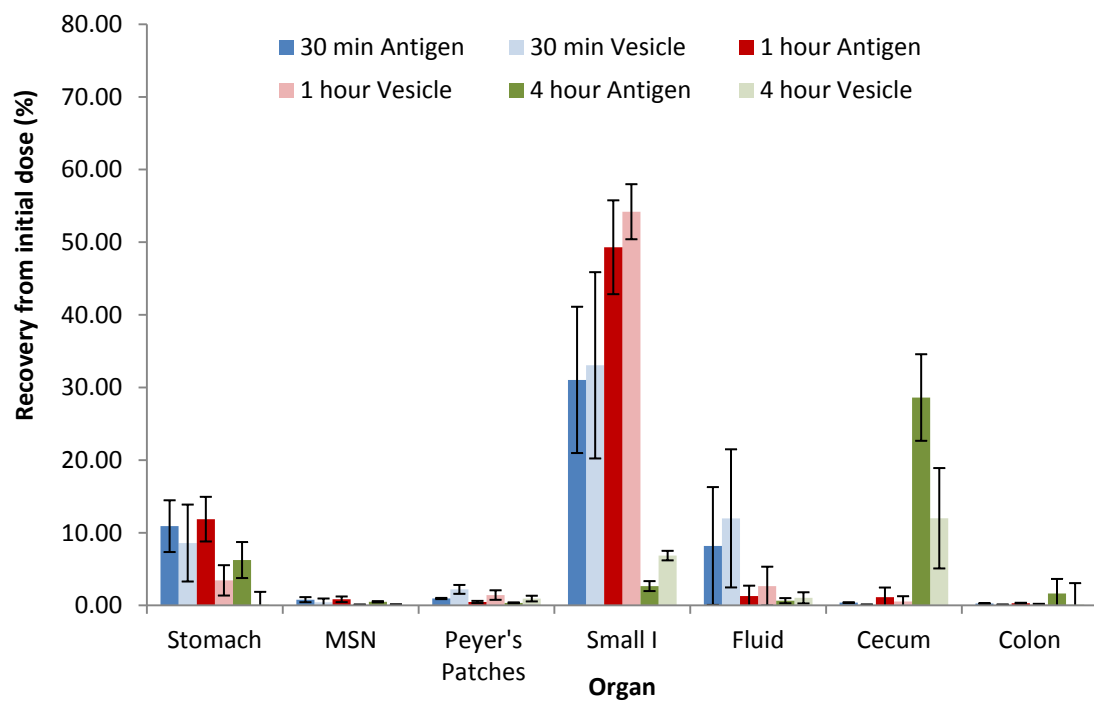
The overall recovery of formulated antigen decreases as the antigen loaded vesicles undergo their transit within the GIT from 30 minutes to 4 hours with levels dropping from 58 % (Figure 5.6A) to 55 % (Figure 5.6B) to finally 44 % (Figure 5.6C). However, for the unformulated antigen in all of the organs collected after 1 hour, recovery of free antigen (38.5 ± 5.6 %; Figure 5.6B) was significantly ($p < 0.01$) lower than antigen formulated within Aston niosomes (59.8 ± 6 %), Aston bilosomes (67.7 ± 7 %) or Strathclyde bilosomes (50.5 ± 7 %) respectively (Figure 5.6B). In particular, the free antigen within the small intestine (5.8 ± 1.8 %) resulted in significantly ($p < 0.01$) lower levels compared to antigen delivered in Aston niosomes (25 ± 10 %), Aston bilosomes (49 ± 18 %) or Strathclyde bilosomes (30 ± 10 %) (Figure 5.6B). Figure 5.6C after the 4 hour time point reflects the transit of the vesicles within the GIT, where the antigen has translocated from areas within the GIT such as the stomach and small intestine to show increased levels of recovery within the colon and cecum which is the latter part of the GI system. Overall, these results shows that the vesicles entrapping the antigen offer longer residence time of the antigen within the GIT ($t=1$ h) and thus do not transit along the GIT to the cecum or colon at a faster rate compared to administering the free antigen dose alone.

Considering between the formulations, there was no significant difference in the antigen recovery in most of the organs at the various time points (Figure 5.6) the niosome formulation showed antigen presence within the cecum after 1 hour which was not apparent for the two bilosome preparations. The presence of antigen within the cecum ($t=1$ h) from the niosome preparation can be correlated with the *in vitro* GI media transit study carried out in section 5.3.2 where the niosome vesicles showed a resultant loss in antigen content of ~ 13 % when in the gastric media (Figure 5.4). The loss of antigen from the vesicles mimics the transit of the free antigen dose where after 1 hour

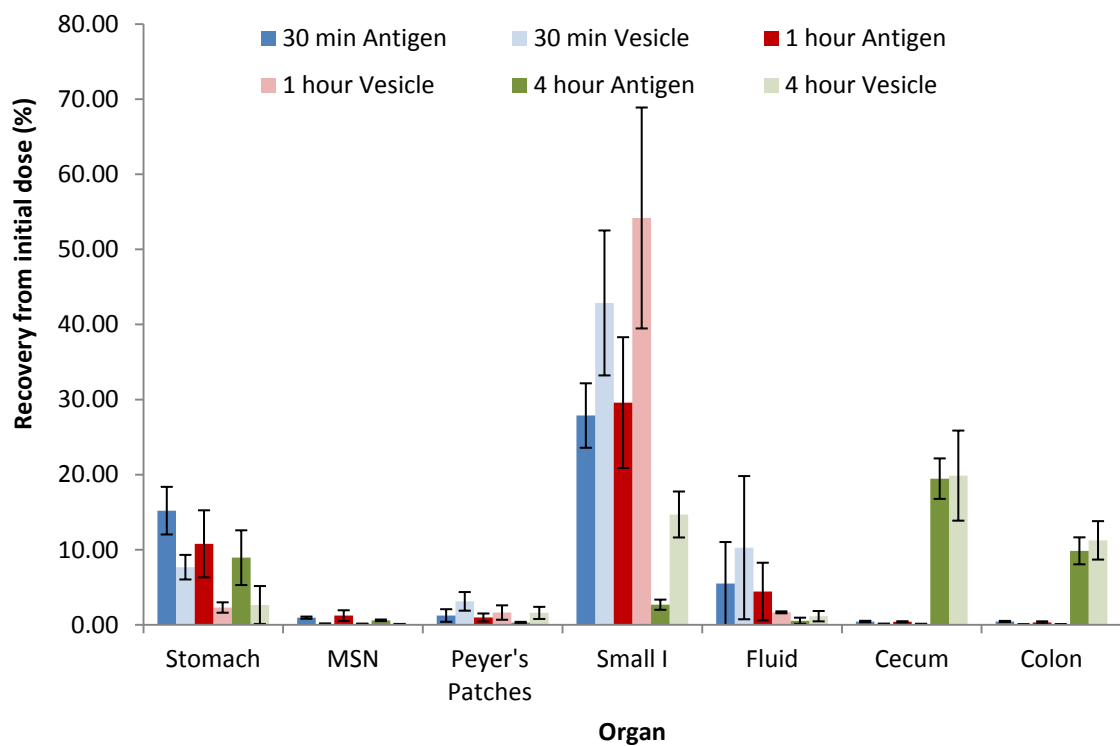
~ 7.4 % of the free antigen dose was recovered from the cecum and ~ 8 % of the antigen from the niosome dose was recovered compared to no antigen from the bilosome preparations.

The antigen recovery alongside the vesicle recovery is presented together in figure 5.8 to determine the overall trend whether the antigen remains with the vesicles along the GI transit. In general, when comparing the vesicle and antigen recovery for all of the organs together (Figure 5.7) the results show that the antigen stays with the vesicles and that the results are comparable between the bilosomes and niosomes at the time points considered. The vesicles in all three formulations (Figure 5.7) within the stomach decrease up to the 4 hour time point which shows that vesicles remain within the stomach for a period of up to 4 hours with vesicle recovery significantly decreasing ($p < 0.05$) from ~10 % ($t = 30$ min) to ~2 % ($t = 4$ h). The only exception to this general trend is that the vesicle recovery (~6 - 14 %) in the small intestine at 4 hours is significantly higher ($p < 0.05$) than the antigen recovery (~2 %). A possible suggestion could be that the vesicles may be adhered within the mucous of the intestine and potential degradation of the vesicles from the various enzymes within the mucous thus releasing the antigen from the vesicles.

A



B



C

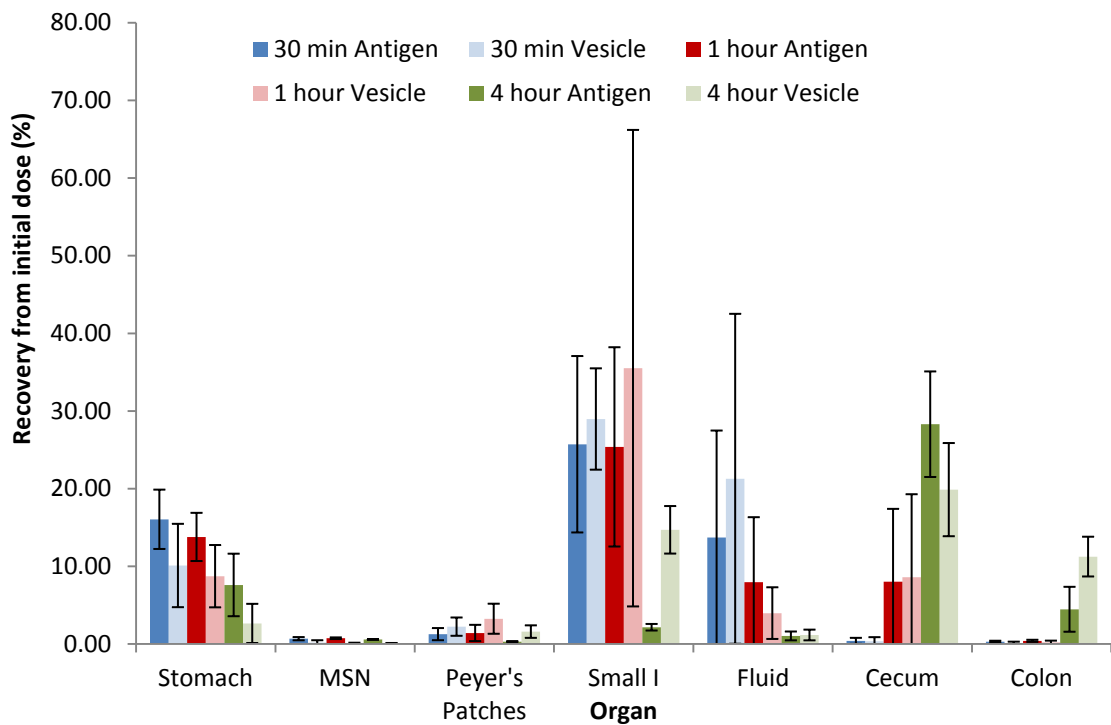


Figure 5.7: A) Aston bilosome preparation showing antigen and vesicle recovery, B) Strathclyde bilosome preparation showing antigen and vesicle recovery and C) Aston niosome preparation showing antigen and vesicle recovery (n=4).

As previously shown, after 1 hour antigen delivered with the niosomal vesicles is recovered within the cecum, compared to the two bilosome preparations. Once again, this could be due vesicle breakdown, and translocation to the cecum at a faster rate than the bilosome vesicles which is confirmed by the recovery of vesicles within the cecum after 1 hour (Figure 5.7C). Studies by Bhavsar and Amiji, (2007) show that radiolabelled gelatin nanoparticles were rapidly degraded within the stomach and small intestines due to the presences of proteolytic enzymes thus rapidly clearing ~85 % of the dose from the GIT after 1 hour (Bhavsar and Amiji, 2007a). However, upon incorporation of the nanoparticles-in-microspheres (NiMOS) of less than 5 μm in size, more than 50 % of the dose was recovered within the small intestine indicating that the microspheres were stable and offered longer residence time within the GIT (Kriegel et al., 2012, Bhavsar and Amiji, 2007a). In addition, they also found that trace levels of

radiolabelled gelatin nanoparticles were recovered from organs such as the blood and liver indicating minimal systemic absorption of particles (Bhavsar and Amiji, 2008, Bhavsar and Amiji, 2007a). These findings are comparable to the findings within our study where un-associated antigen is rapidly cleared to the cecum and colon after 1 hour (Figure 5.6B) with antigen recovery levels of 5 % within the small intestine compared to an average of 40 % antigen recovery within the small intestine when associating the antigen with vesicles.

Within the Peyer's patches, the vesicle recovery between 1 hour and 4 hours for all formulations showed no significant differences; however, the antigen retention within the Peyer's patches significantly decreased from 1 hour to the 4 hour time point suggesting that antigen is migrating away from the vesicles (Figure 5.8A). This effect has also been observed by Henriksen-Lacey et al, (2010) where a biodistribution study using charged DDA:TDB liposomes were injected intramuscularly were shown to retain vesicles at the site of injection for a period of 4 days with no significant change however, antigen content decreased significantly from days 1 to 4 whereas increased antigen levels were found in the draining popliteal lymph nodes (Henriksen-Lacey et al., 2010b). Henriksen-Lacey et al, (2010) also confirm that the charged liposomes are retained for longer periods of time at the site of injection and that antigen adsorbed to the vesicles also offer better retention (Henriksen-Lacey et al., 2010b). The increased bioavailability and antigen retention is also confirmed within this study, where the association of antigen with niosome/ bilosome vesicles resulted in significantly higher ($p < 0.05$) vesicle/antigen recovery levels compared to administration of the free antigen dose, which was rapidly cleared from the GIT. Therefore, this study confirms the advantage of associating antigen with niosome/bilosome vesicles as antigen is protected

from the external milieu of the GIT and in turn offers increased residence time of the vaccine within the GIT.

5.3.5 Peyer's patch targeting

Within the mouse model, there were approximately 8 to 11 Peyer's patches recovered which are in line with literature (6-12 patches; (Yeh et al., 1998)). In terms of targeting to the Peyer's Patches, figure 5.8A shows that there was no significant difference between uptake and retention of the free antigen and antigen entrapped within the vesicles. The Peyer's patches are located at the antimesenteric border of the intestine where they appear as nodular white masses (1.5 – 3.0 mm) and are key to antigen uptake and induction of immunity (Yeh et al., 1998). The Peyer's patches are an attractive route for targeted delivery as this provides a direct route for antigen to reach the lymphatic system where first pass metabolism is avoided and the chances of cellular rejection are reduced due to the M cells not expressing P-glycoprotein efflux pumps (Hunter et al., 2012, Florence, 1997). Furthermore the Peyer's patches, in comparison to the rest of the GI mucosa, possesses minimal mucosal coating where transcytotic activity is high (Frey et al., 1996, Plapied et al., 2011). The results show that the uptake of vesicles within the Peyer's patches showed uptake within the range of 1-5 % (Figure 5.8A) and after studying the vesicle size after exposure to simulated GI fluids, the vesicles still remain within the size range 3-10 μm which are suited for uptake and retention within the Peyer's patches. Furthermore, the study presented in section 5.3.5 has also shown that vesicles within this size range show increased uptake and retention compared to vesicles of a smaller size range of 2 μm .

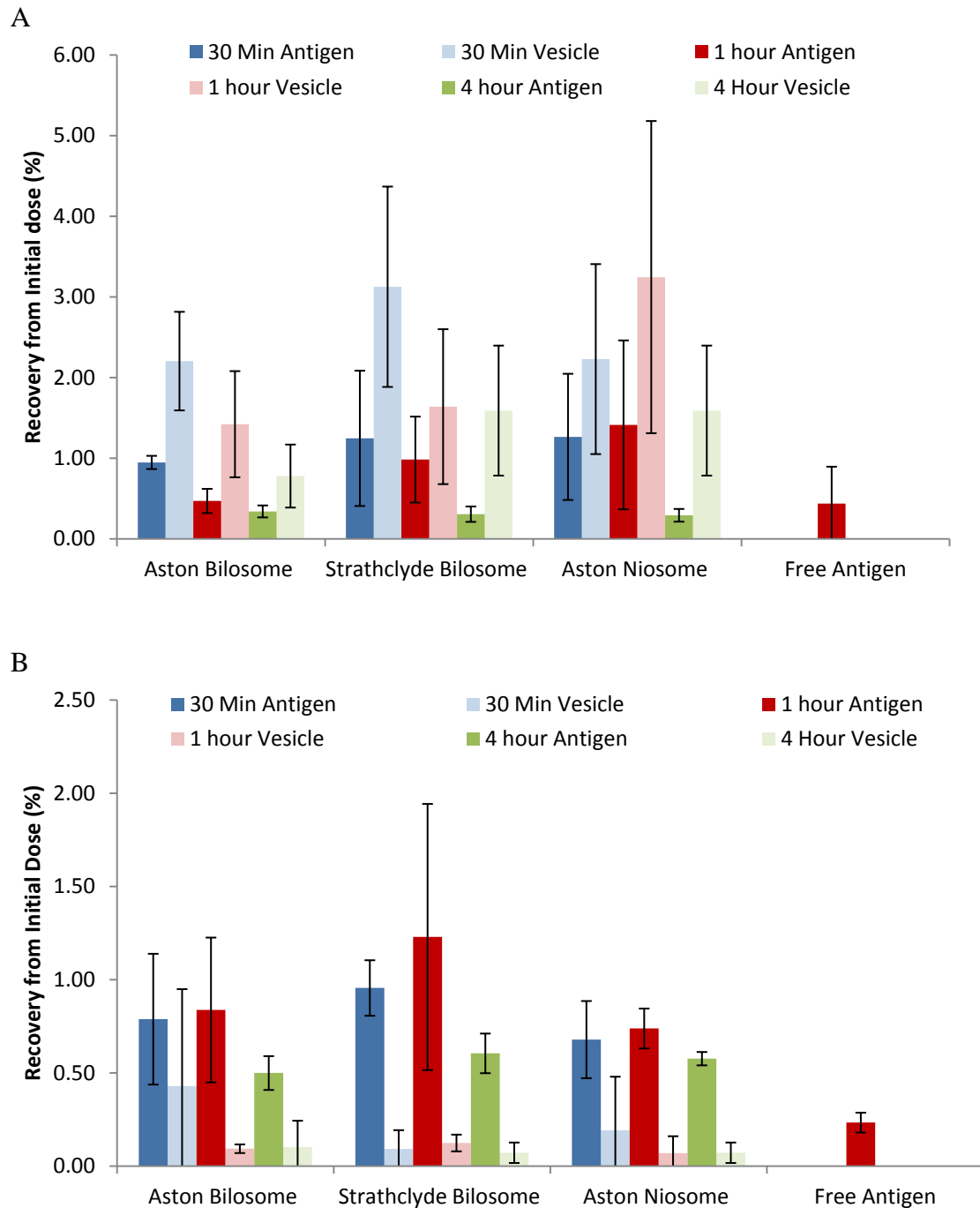


Figure 5.8: Recovery of antigen and vesicles based on initial dose within **A)** Peyer's patches and **B)** Mesenteric Lymph Tissue (n=4).

Antigen recovery within the Mesenteric lymph tissue as presented in figure 5.8B for the niosome/bilosome formulations is significantly greater ($p < 0.01$) than that of the free antigen administered dose. The results show that even after 4 hours the antigen recovery of the niosome/bilosome vesicles within the mesenteric tissue is still greater than the

recovery of free antigen after 1 hour. The data indicates that the vesicles and the antigen are able to penetrate and reside within the lymph tissue for a period of 4 hours without any change in the recovery levels. This is beneficial as the vesicles which remain in the Peyer's patches offer increased mucosal immunity whereas the increased antigen and vesicle within the mesentery is more likely to induce systemic immunity, thus offering both mucosal and systemic immunity (Tabata et al., 1996). In addition, Neutra and Kozlowski (2006) determine that antigens delivered to mucosal sites within the small intestine offer greater IgA secretions which are a characteristic of mucosal immunity. In comparison to antigen delivery via routes such as the nasal route promote a systemic antibody response, due to the migration of antigen to the draining lymph nodes (Neutra and Kozlowski, 2006).

In terms of comparing niosomes and bilosomes, there were no significant differences between the uptake within the Peyer's patches and the mesenteric lymph tissue (Figure 5.8). This prompted further studies to consider the impact of dose concentration on antigen recovery within the Peyer's patches.

5.3.5.1 *In vivo* Saturation study with bilosome preparation

Given that the biodistribution study in section 5.3.4 showed that uptake in the Peyer's patches and mesenteric lymph tissue remained constant and then decreased slightly over the time period from 30 minutes to 4 hours (Figure 5.6, Figure 5.8A), a question arose; is uptake by the Peyer's patches limited in terms of saturation of antigen or vesicle uptake? To address this a study considering varying doses of the vaccine was carried out which involved four groups of a) double dose (antigen 180 µg/mL, lipid 27 mg/mL), b) normal dose (antigen 90 µg/mL, lipid 13.5 mg/mL), c) half dose (antigen 45 µg/mL, lipid 6.75 mg/mL) and d) quarter dose (antigen 22.5 µg/mL, lipid 3.375

mg/mL) was designed to determine uptake of vesicles and antigen throughout the GIT in particular attention to the target site Peyer's patches and Mesenteric lymph tissue. Based on the biodistribution study the organs removed for the following study focussed specifically on the GI tract which included the stomach, Mesenteric lymph tissue, Peyer's patches, small intestine, colon and cecum, where the data is presented for antigen and vesicle in figure 5.9.

Results from the study (Figure 5.9) show no significant differences in percentage antigen recovery or vesicle recovery between different dose concentrations in the organs collected after a 30 minute time point (Based on each dose initial counts). The overall percentage recovery of antigen is comparable (40-70 %) between all doses administered based on the initial dose count (Figure 5.9A). The vesicle recovery (Figure 5.9B) between the organs when changing dose concentrations is also comparable with no significant differences suggesting that the clearance rate and the gastric emptying time is not formulation dependent. Gastric emptying time has been attributed to the fed or fasted state of the GIT, where the gastric emptying time $T_{1/2}$ in the fasted state is up to 2 ± 1 min compared to a fed state $t_{1/2}$ of 17 ± 2 min (Roda et al., 2010). However to consider if the amount of lipid and antigen was influenced by the dose given, the results were also analysed in terms of mass of antigen and lipid (Figure 5.10).

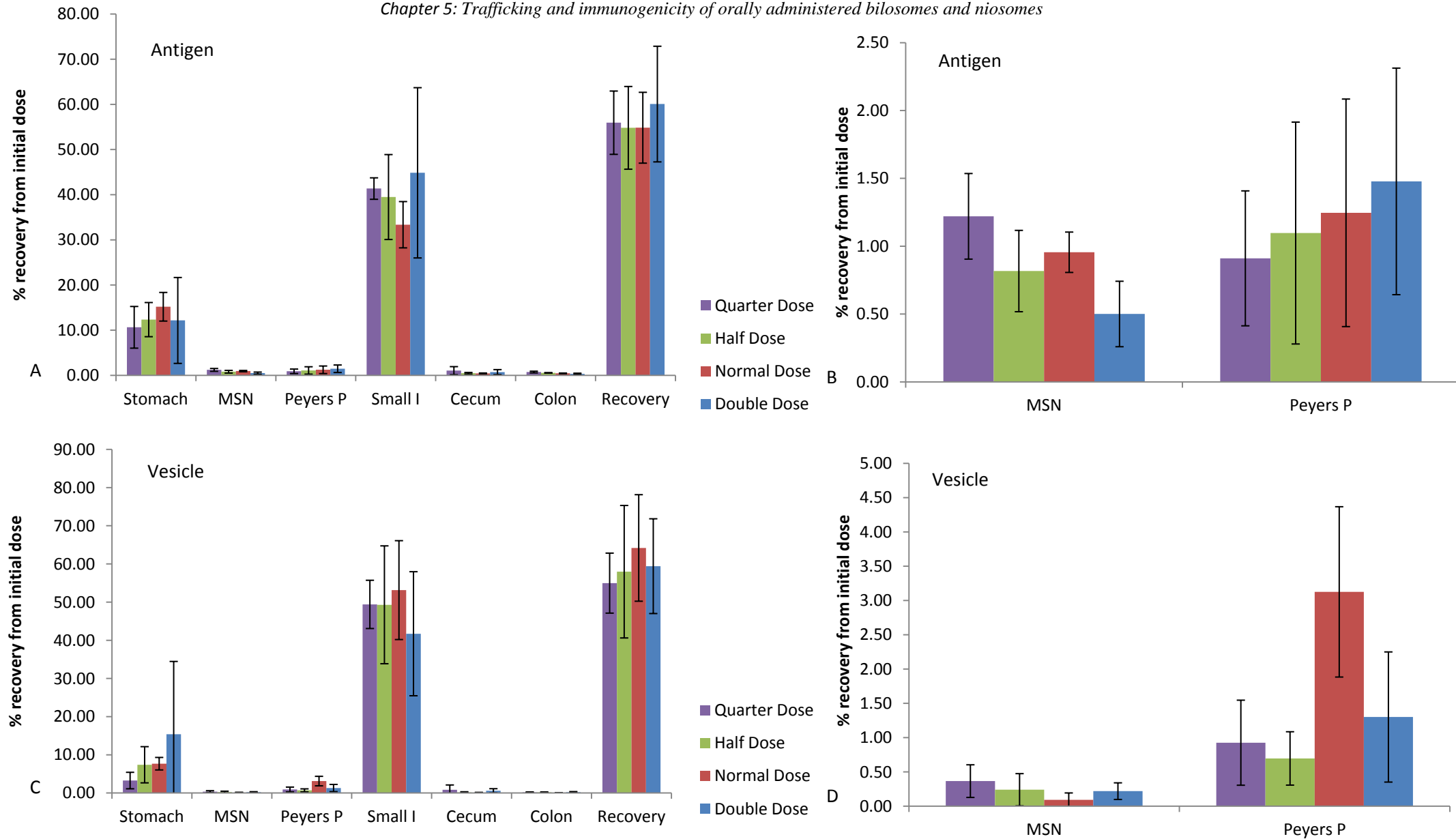


Figure 5.9: *In vivo* saturation study of various dose concentrations representing antigen and vesicle recovery within the organs selected. **A)** Antigen recovery, **B)** Mesenteric tissue and Peyer's Patches antigen recovery, **C)** Vesicle carrier recovery and **D)** vesicle carrier recovery within the Mesenteric tissue and the Peyer's patches (n=4).

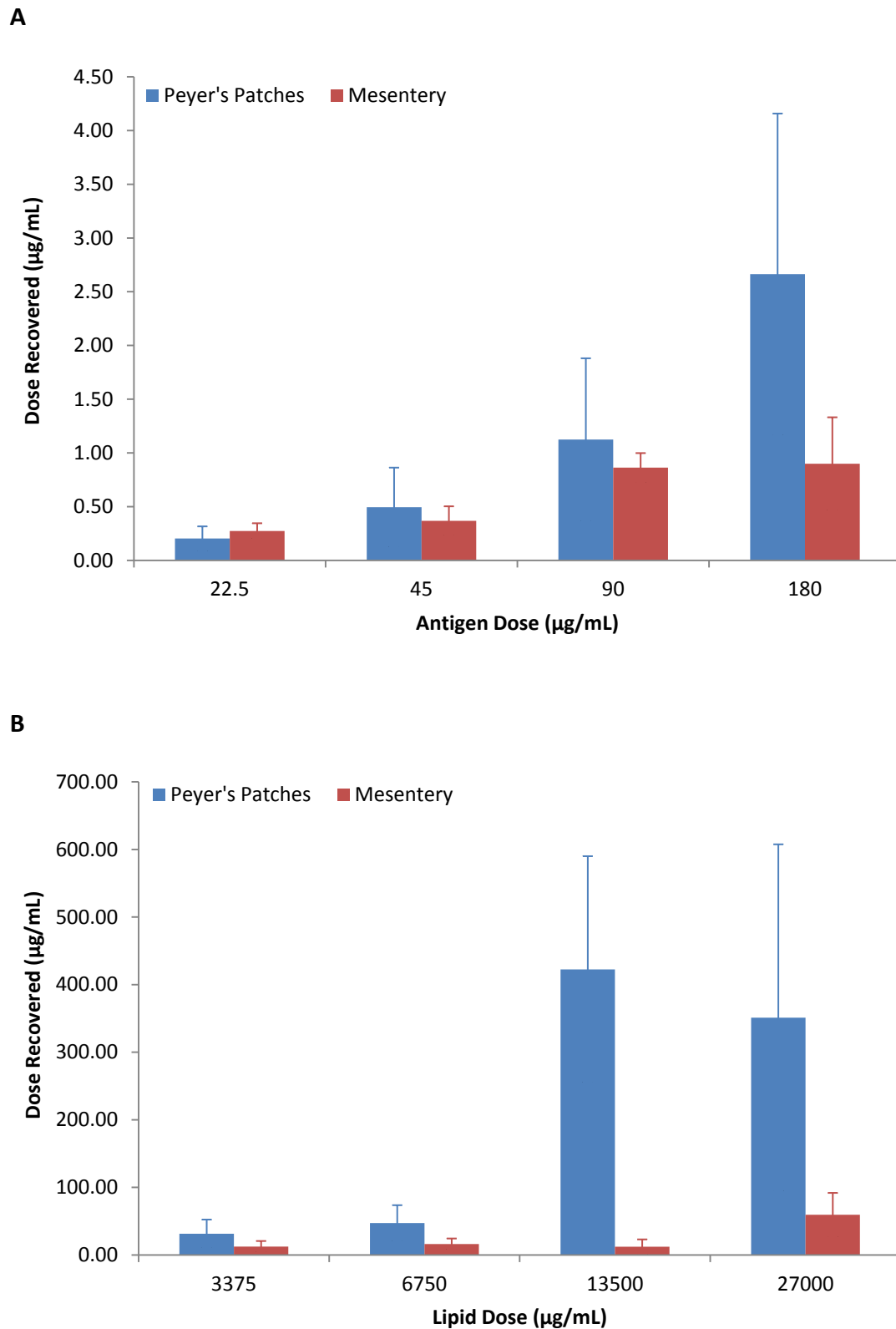


Figure 5.10: Representation of dose recovery per mL at site of uptake for various dose concentrations of the formulation within Peyer's patches and mesenteric lymph tissue. **A)** Antigen dose and **B)** Lipid dose. (n=4).

A closer look at the Peyer's patches and mesentery in figure 5.10A shows that the higher the antigen dose administered the higher the antigen recovery levels within the organs however, there was no significant difference between the double dose and normal dose administered. Hence suggesting that saturation point is being reached within the Peyer's patches and mesentery which is confirmed by figure 5.10B where the increase in lipid dose also shows no significant difference between the normal and double dose. This effect of saturation based on dose has been observed by Nograles et al, (2012) where 50, 100 and 150 µg doses of pDNA-loaded alginate microspheres carrying a green fluorescent protein (GFP) were orally administered. Upon the delivery of a plasmid DNA vector to determine GFP expression (Intestinal cells) 24 hours post administration, the 100 µg dose yielded higher GFP expressing cells compared to a 50 µg dose. However, saturation had been reached as the increment of dose to 150 µg yielded lower GFP expressing cells than the 50 µg dose suggesting dose controlling is important (Nograles et al., 2012). Therefore, controlling dose is vital as a strategy of increasing dosing could potentially lead to reduced secretion of antigen specific IgA levels, due to increased systemic tolerance to the vaccine (Mowat, 2003).

5.3.6 Size reduction implications on uptake within GIT for bilosomes

To consider the impact of vesicle size on biodistribution vesicles were prepared as mentioned in section 5.2.3 where bilosomes were produced using the Strathclyde method. The vesicles were then probe sonicated for two periods of 30 seconds to reduce the overall VMD of the vesicles and were then centrifuged to remove the un-entrapped antigen and then administered to 5 female Balb/c mice.

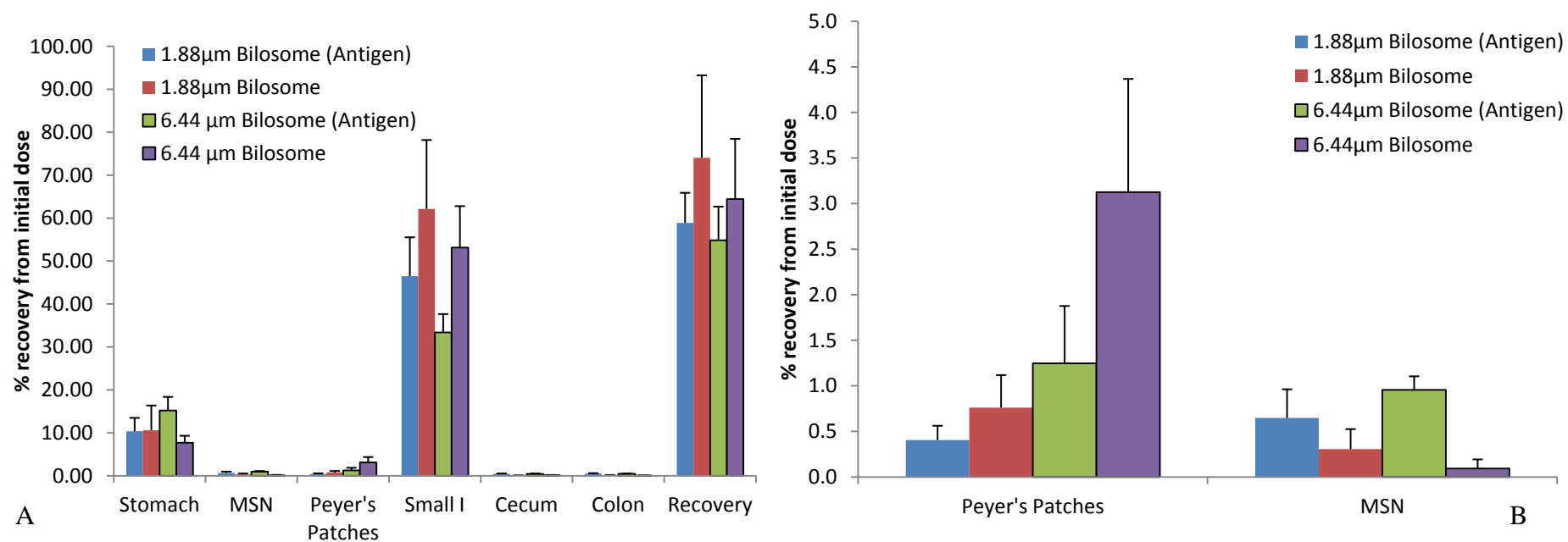
Table 5.2 shows that the reduction in vesicle size was successful and that an overall smaller range of vesicles was produced. These samples then underwent an *in vivo*

biodistribution study and were compared to a normal dose in the previous study in section 4.3. The antigen and lipid recovery data suggests that the majority of the formulation after 30 minutes is present within the small intestine, with approximately 10 % in the stomach. These results (Figure 5.11A) are in line with previous studies where the vesicles were un-sonicated and show good overall recovery (Figure 5.9B). When looking at the Mesenteric lymph tissue, the results from the two formulations shows no significant differences in the antigen uptake (Figure 5.11B); however when looking at the Peyer's patches, antigen recovery is significantly ($p < 0.05$) higher when delivered with the larger vesicle population. There is also a greater recovery of vesicles within the Peyer's patches of the larger vesicles in comparison to the sonicated smaller vesicle formulation (Figure 5.11B).

This would suggest that it is advantageous to keep the larger vesicle sized formulations as the vesicles which remain in the Peyer's patches offer increased retention time and hence can stimulate the dendritic cells to produce the effective immunity. Considering the target site uptake of antigen and carrier into the Peyer's patch and mesenteric lymph tissue (Figure 5.11B), significantly higher ($p < 0.01$) levels of the larger vesicles was found in the Peyer's patches compared to the smaller vesicle size formulation (Figure 5.11B). However this did not translate into significantly increase the levels of bilosomes or antigen in the mesentery. A similar trend has been observed by Ebel (1990), who compared the uptake of 9 μm and 2 μm polystyrene latex beads via the Peyer's patches and mesenteric lymph nodes. The results showed that the larger particles retained within the Peyer's patches with no presence in the mesenteric lymph nodes, whereas the smaller particles were more noticeable in the mesenteric lymph nodes (Ebel, 1990).

Table 5.2: Overall VMD and size distribution of the administered vesicles.

	Vesicle Size (μm)	Span
No Sonication	6.44 ± 0.05	1.75
Sonication	1.88 ± 0.37	2.45

**Figure 5.11:** Influence of vesicle size: **A)** *In vivo* biodistribution data for antigen and vesicle (tritium) for the organs selected from the GIT and **B)** A closer look into the mesenteric lymph tissue and Peyer's patches for the smaller vesicle formulation and for the normal sized formulation (n=4).

Furthermore, in terms of immune response, this trend has also been observed by other studies where, a summary by Oyewumi et al, (2010) also suggests that in terms of oral vaccine delivery using lipid vesicles, the larger vesicle sizes (400–2500 nm) induced significantly higher IgG2a titres and IFN- γ responses than the smaller vesicles (Oyewumi et al., 2010). A study directly comparing immune responses to antigens delivered orally with 200, 500 or 1000 nm PLGA particles showed the largest particle group to be the most effective, suggesting that although the smaller particles would be more readily absorbed, their lack of targeting to the Peyer's patches reduces their immunogenicity (Gutierrez et al., 2002). In addition, studies using PLA for oral delivery of vaccines have shown that the optimum vesicle size for induction of an IgG response is 4 μm . Moreover, Eldridge et al, (1990) noted that uptake was restricted to particles less than 10 μm . However, whilst particles between 5 and 10 μm remained within the Peyer's patches, the majority of microspheres less than 3 μm were transported through the efferent lymphatics within macrophages, suggesting that this pattern of absorption and redistribution may determine the type of immune response elicited by the vaccines (Eldridge et al., 1990). Within their paper they predict that microspheres below 3 μm would induce a predominantly circulating antibody response, while those above this size would stimulate a mucosal (IgA) immune response. Mladenovska et al, (2002) further confirm that their 7 μm BSA loaded gelatin microspheres were adsorbed through the M cells into the Peyer's patches where higher local antigen concentration was observed and upon decreasing particle size, higher recovery of BSA loaded microspheres was found in the liver and spleen via the mesenteric lymph nodes (Mladenovska et al., 2002). Thus, the results obtained from the biodistribution study of the unsonicated formulations (VMD- 6.44 μm) which show increased vesicle and antigen recovery within the Peyer's patches offer a greater chance to increase mucosal

immunity. This study confirms that it is important to understand the vesicle size characteristics in relation to the desired site of uptake depending on the route of delivery thus potentially influencing the type of immunity produced.

5.4 Conclusions

The proposed method of producing vesicles to protect thermolabile antigens by lowering the process temperatures (Aston melt) shows that antigen can be associated and entrapped within non ionic surfactant vesicles. Confocal laser scanning microscopy confirmed that the vesicles are spherical in nature and the vesicles meet the criteria of being within the optimum size range and charge for increased uptake within the target site of the Peyer's patches. On average 1.5 % antigen is taken up by the Peyer's patches based on initial dose administered after 30 minute time point. This is the highest point of uptake within the Peyer's patches, after this time antigen levels stay the same or decrease over time. Based on the saturation studies, the dose concentration can be increased but once again only an average of 1.5 % antigen (based on dose) is recovered within the Peyer's patches and although an increase in antigen concentration is calculated this is not significantly different to the normal dose that would be administered. The presence of the vesicles show increased retention of antigen within the mesenteric lymph tissue and overall recovery during transit compared to administering free antigen alone thus increasing the chances of uptake. To conclude, this study shows that it is important to control and understand dosing and antigen concentrations administered as increased dose concentration would not be commercially viable with the higher chance of reduced immunity due to oversaturation and increased systemic tolerance. Furthermore, larger vesicles are able to reside within the Peyer's patches compared to the corresponding smaller vesicles thus is an indicator of providing increased mucosal immunity.

Chapter 6

Development of an alternative dosage platform for oral
vaccine delivery using freeze drying

6.0 Introduction

Oral liquid vaccines have previously been shown to enhance immunity and show comparative data to commercial vaccines when administered intramuscularly (Chapter 2). In the development of an oral vaccine generally the dosage form would be in a liquid state. However, in terms of long term shelf stability, storing vaccines as liquid dosage forms is not ideal due to stability issues. Hence, the development of a solid dosage vaccine platform which was initially formulated as a liquid is the desire in overcoming stability and storage issues.

Freeze drying has been widely used as a standard method for enhancing the stability of liposomal preparations. Freeze drying of liposomes is used to prevent hydrolysis and physical degradation of the phospholipids used within the vesicles during extended storage (van Winden, 2003, Bridges and Taylor, 2001). However, the process of freezing and resultant dehydration of the formulation can exert stress onto the vesicles thus affecting the integrity of the vesicles. Freezing may result in ice formation thus disrupting the bilayers and result in phase transition changes (Stark et al., 2010). Upon dehydration, an increase in solute concentration can occur which may initiate chemical destruction of vesicles thus losing integrity, causing bilayer fractures, subsequently leading to vesicle aggregation, changes in vesicle size, and loss of entrapped antigen/material (Crowe et al., 1985, Crowe et al., 1986, Stark et al., 2010).

The associated problems with freeze drying of bilayered vesicles can be minimised by the inclusion of cryo- and lyoprotectants which include disaccharide carbohydrate sugars within the formulation prior to freeze drying. Protectants such as trehalose or sucrose are characterised by their non-eutectic nature, thus protecting the vesicles by forming an amorphous matrix around the vesicles. The addition of trehalose to a vesicle suspension inhibits vesicle fusion and aggregation during the freezing process

(Abdelwahed et al., 2006, van Winden et al., 1997). The mechanism of action of such cryoprotectants has been examined by Crowe et al, (1996) where they found that the sugar molecules are able to interact with the head groups of the phospholipids, thus, preventing membrane disruption by counteracting fusion (Crowe et al., 1996). In addition to adding a cryoprotectant to the suspensions, other excipients have also been known to aid in the lyophilisation process offering protection of the product (Table 6.1).

Table 6.1: Excipients and their functions within the lyophilisation process.

Parameter	Function	Example excipients
Bulking agents	When concentration of product is low it is useful to add bulking agents to freeze-dry the system.	Hydroxyethyl starch, trehalose, mannitol, lactose, and glycine
Buffers	Some buffers alter pH after freeze-drying	Phosphates, Tris, citrates
Stabilisers	Protect product through freezing stages	Sucrose, lactose, glucose, trehalose, glycerol, mannitol, sorbitol, glycine, alanine, lysine, polyethylene glycol, dextran, and polyvinyl pyrrolidone
Isotonicity modifiers	Controls isotonicity	Mannitol, sucrose, glycerol
Increase collapse temperature	Allows higher drying temperatures	Dextrans, PEG, PVP,

The oral route offers increased patient compliance and the possibility of providing herd immunity is increased if the vaccine is easily administered. Tablets and capsules are considered to be dosage forms which offer the high patient compliance. In addition there are options of fast melt tablets for paediatrics, geriatrics, patients who may struggle with swallowing (after stroke or renal failure patients) or patients with dysphasia (Lindgren and Janzon, 1991, Wilson et al., 1987, Gupta, 2010). In terms of vaccine solid dosage forms, this resolves the issue of storage and is a more cost effective way to carry out bulk immunisation as tablets can be distributed worldwide without the use of trained personnel.

6.1.1 Aims and Objectives

The aim of this project was to exploit excipients used for lyophilisation (Table 6.1) to formulate an oral vaccine solid dosage platform which, upon rehydration or ingestion releases the vesicles containing the antigen. These vesicles should also maintain their integrity, potency and function as they would in a liquid dosage form. The studies involved:

1. preparing a powdered sachet which a user could mix with water prior to administration;
2. producing a fast melt tablet that, upon contact with water immediately redisperses to form vesicles. This table could either be placed in some water to drink or directly into the mouth;
3. producing an oral chewable dosage form which will release contents within the mouth and expose the vesicles to mucosal sites along the Mucosa associated lymphoid tissue;
4. developing a vaccine capsule which protects the contents against the stomach acids and then release the contents closer to or within the small intestine.

6.2 Materials and Methods

To form the vesicles the surfactants Monopalmitoyl glycerol (MPG; Larodan AG, Sweden), Synthecol (Chol), Dicetyl phosphate (DCP) (Sigma-Aldrich, UK) were used. The buffers were made up of sodium bicarbonate (Sigma-Aldrich, UK) at pH 7.6, where hydrochloric acid and sodium hydroxide (NaOH) (Sigma-Aldrich, UK) was used for pH adjustments. For the antigen a recombinant H3N2 sub-unit protein (Immune Tech, USA) was used.

6.2.1 Preparation of niosomes for lyophilisation

A 5:4:1 molar ratio of MPG, Chol and DCP was weighed and placed into a 10 mL glass beaker ensuring that no lipids stick to the sides of the beaker. Before melting the lipids

the aqueous phase was prepared in a 15 mL falcon tube. 25 mM sodium bicarbonate (pH 7.6) formed the aqueous phase and was then placed in a heated water bath for 10 minutes at 30-35 °C. Whilst the aqueous buffer was preheated, the beaker containing the lipids was placed into a hot oil bath (120-125 °C) and melted for 10 minutes with occasional mixing. The beaker containing the molten mixture was removed from the oil bath and the buffered stock solution was immediately added and homogenised at 8000 rpm at 30-35 °C. After homogenising for 10 minutes, the homogenisation speed was reduced to 4000 rpm to act as a mixer and dextran (Sigma Aldrich, UK) was added and mixed into the solution for a minute followed by mannitol (Sigma Aldrich, UK) for another minute of mixing.

A 400 mM trehalose solution was prepared and then added 1:1 of niosome mixture: trehalose in a bijoux tube which formed the mould for the tablets and were then pre-frozen in the -70 °C freezer until the freeze drying cycle was ready to begin.

6.2.2 Lyophilisation cycle

Lyophilisation was performed with the Virtis Advantage (Bio Pharma) freeze dryer. The samples to be freeze dried were stored at -70 °C and the freeze drying protocol was set for primary drying to occur at -40 °C for 48 hours with a secondary drying cycle set at 20 °C for a further 10 hours with a condenser temperature set at -75 °C. The freeze drying cycle was sufficient enough to thoroughly dry the formulations and prevent damage to the samples.

6.2.3 Freeze fracture microscopy of niosome vesicles

A 5 µL drop of each incubation mixture was placed on a ridged, gold specimen support or was sandwiched between two copper plates for fracture in a double replica device. Samples were frozen by rapid plunging into a constantly stirred mixture of

propane:isopentane (3:1) cooled by liquid nitrogen. Fracture was performed on a Balzers BAF 400D apparatus at a temperature of -110°C . Replicas were floated free on distilled water and cleaned in 40 % chromic acid. Images were then viewed using a Jenway transmission electron microscope.

6.2.4 TGA of freeze dried tablet samples

A small sample of the tablets were broken and the sample was placed onto the Perkin Elmer TGA apparatus and was weighed and then analysed. The method set, heated the sample to 110°C and moisture content was determined as a % weight loss of the sample. All samples were repeated in triplicate to determine moisture content and degradation. All formulations were carried out using nitrogen and air as the purge gasses.

6.2.5 Mechanical strength

The mechanical properties (Hardness) of the tablets were analysed by a Tinius Olsen texture analyser (Hounsfield, UK) equipped with a 50 N load cell. The instrument was calibrated with standard weights and the tablets were placed individually on a platform. The hardness was expressed as the peak force (N) after a 2 mm penetration of a 5 mm diameter probe at velocity of 6 mm/min was applied. The average of five batches was taken as replicates.

6.2.6 Disintegration time

Disintegration time was measured using the USP apparatus (Erweka, ZT3) test for disintegration where 800 mL distilled water was kept at constant temperature of 37°C . A basket with a wire mesh was raised up and down within this 800 mL media at an interval of 30 cycles/ min. Samples were run individually and were tested in triplicate.

6.2.7 Determination of vesicle size and zeta potential

The vesicle size distribution was determined using laser diffraction on a sympatec 2005 (Helos/BF) cuvette analyser. 20 μL of the niosome suspension was diluted into the cuvette with 40 mL double distilled water. The zeta potential which is an indirect measurement of the vesicle surface charge, was measured in 1.5 mL double distilled water at 25 °C on a Zeta Plus Brookhaven Instrument. 15 μL of the niosome suspension was mixed in 1.5 mL double distilled water and then analysed.

6.2.8 Labelling and quantification of antigen

Initially the H3N2 antigen was incubated with the fluorescent flamma fluor FPR-648 (Bio Acts) marker and a conjugation buffer was added and left at 30 °C for 4 hours to conjugate. After this period the unbound fluorescence was removed by centrifugation through an amicon P-10 centrifugal 10 kD MWCO filter tube. The remaining fluorescent antigen was made back to volume and then a spike of this was used per formulation as listed above. The H3N2 spike was placed into the aqueous phase prior to homogenisation. Initially a calibration curve was constructed on the same plate as the formulations after rehydration and all conditions for all formulations was kept constant (Figure 6.1).

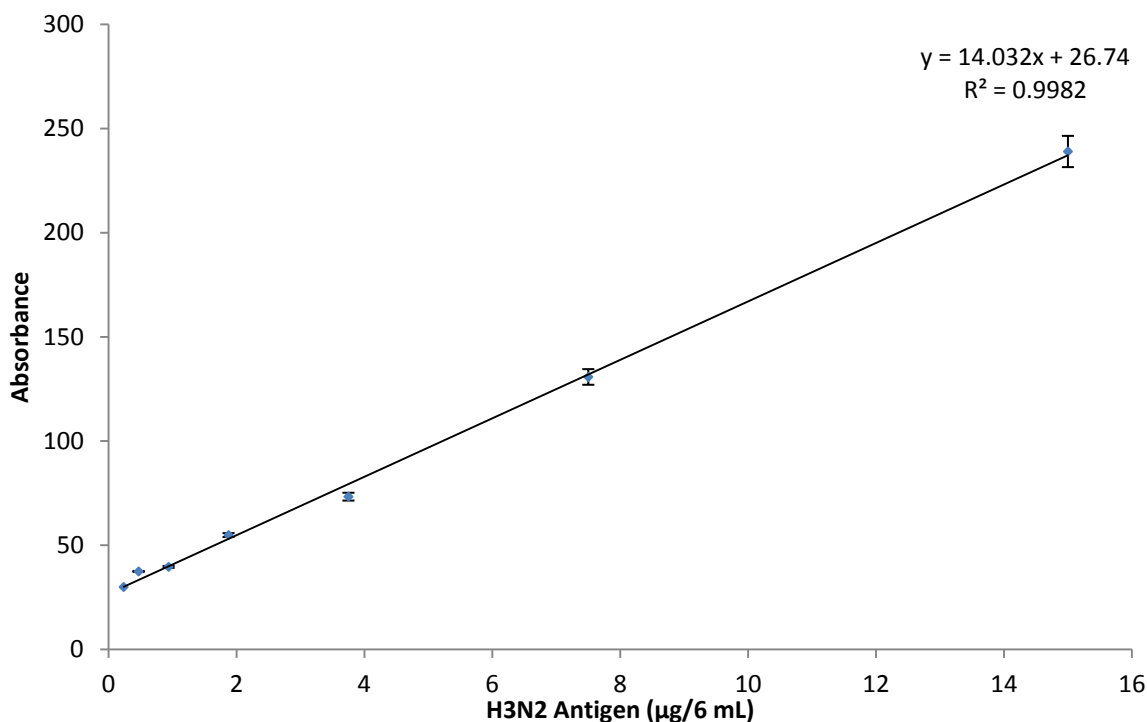


Figure 6.1: Calibration of Fluorescent H3N2 antigen at 648 nm emission and 672 nm excitation. Initial antigen concentration within niosome 6 mL was 13.6 µg (n=3).

Vesicles were prepared incorporating the fluorescent antigen and freeze dried. For quantification of antigen association, ultra-centrifugation of the formulations was required to isolate antigen entrapped vesicles, from non-incorporated antigen. To achieve this, freeze dried samples were rehydrated and 300 µL aliquots of sample were diluted in a Beckman 3.9 mL Polo-allomer tube and filled up to the neck with double distilled water. The tubes were heat sealed ensuring that there were no air bubbles present within the tube. The tubes were then placed accordingly in the TLN-100 rotor and centrifuged at 354,000 X g for 45 minutes at 4 °C with the acceleration set at 8 (Slowest) and the deceleration at coast. When centrifugation was complete, the tubes were opened and the supernatant removed. The sample was resuspended and the centrifugate stage repeated. After the second ‘wash’ the antigen loaded vesicles were then re-suspended with 100-200 µL of appropriate buffer and transferred to a black microplate for reading fluorescence.

6.3. Results and Discussion

6.3.1 Optimisation of freeze drying cycle

It is vital to develop an economical freeze drying cycle, which is dependent on formulation, surface area, freezing process, pressure and temperature. Lyophilisation is a time-consuming and energy intensive process thus optimisation of a cycle is vital (Hilleman and Hurni, 1982). The first step of freeze drying is the thermal treatment of the sample ie, pre-freezing, where the liquid suspension is cooled and ice crystals are formed. The water present within the formulation freezes and forms crystals, thus increasing the viscosity of the suspension. As previously mentioned, the presence of ice crystals may disrupt bilayers and lead to vesicle instability (Stark et al., 2010). In general, fast freezing results in smaller ice crystals being produced compared to slow freezing which results in large crystals and pores. As a result, pre freezing of the liquid suspensions can be carried out by freezing at -70 °C to overcome the issue of producing large vesicles and larger pores. When optimising the freeze drying cycle the sublimation rate of the ice during primary drying is the most crucial stage and this depends upon several factors such as surface area, pressure and temperature. There are four main features of primary drying which have been highlighted and summarised in figure 6.2 (Abdelwahed et al., 2006, Bridges and Taylor, 2001, Pikal et al., 1990). Figure 6.3A and B represents the samples loaded within the freeze dryer containing the thermocouples ready to start the cycle and the sublimation rate is presented in figure 6.3C which demonstrates that the sublimation of the ice crystals takes place from the surface to the bottom of the vessel.

The freeze drying cycle, consists of two phases which include primary drying and secondary drying (Williams and Polli, 1984). Figure 6.3C represents primary drying where sublimation of the ice from the frozen sample is taking place.

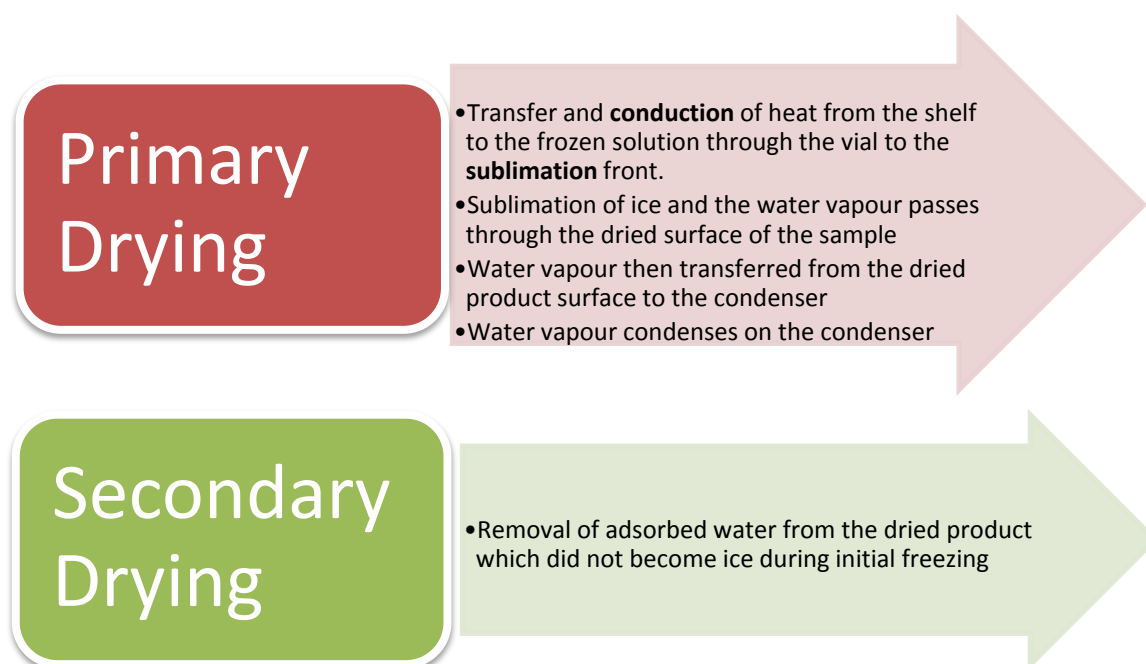


Figure 6.2: Summary of primary and secondary drying during a standard lyophilisation procedure.

Initially, the lyophilisation procedure for lipid based vesicles within this study was obtained from the development of a freeze drying protocol for TB vaccines based on DDA:TDB liposomes within the Aston university group (Mohammed et al., 2006, Mohammed et al., 2007). Within these studies, it was found that PC: cholesterol liposomes at a 16:4 μmol ratio when pre frozen at $-70\text{ }^{\circ}\text{C}$ followed by 10 hours primary drying at $-50\text{ }^{\circ}\text{C}$ with 24 hours secondary drying at $-30\text{ }^{\circ}\text{C}$ was a stable protocol with the inclusion of a cryoprotectant, determined by the vesicle size after rehydration (Mohammed et al., 2006). The preparation of niosome vesicles includes the use of significantly higher lipid concentrations within the formulations compared to liposome vesicles hence, based on the protocol developed by Mohammed et al, (2006) the lyophilisation cycle was further optimised for drying times.

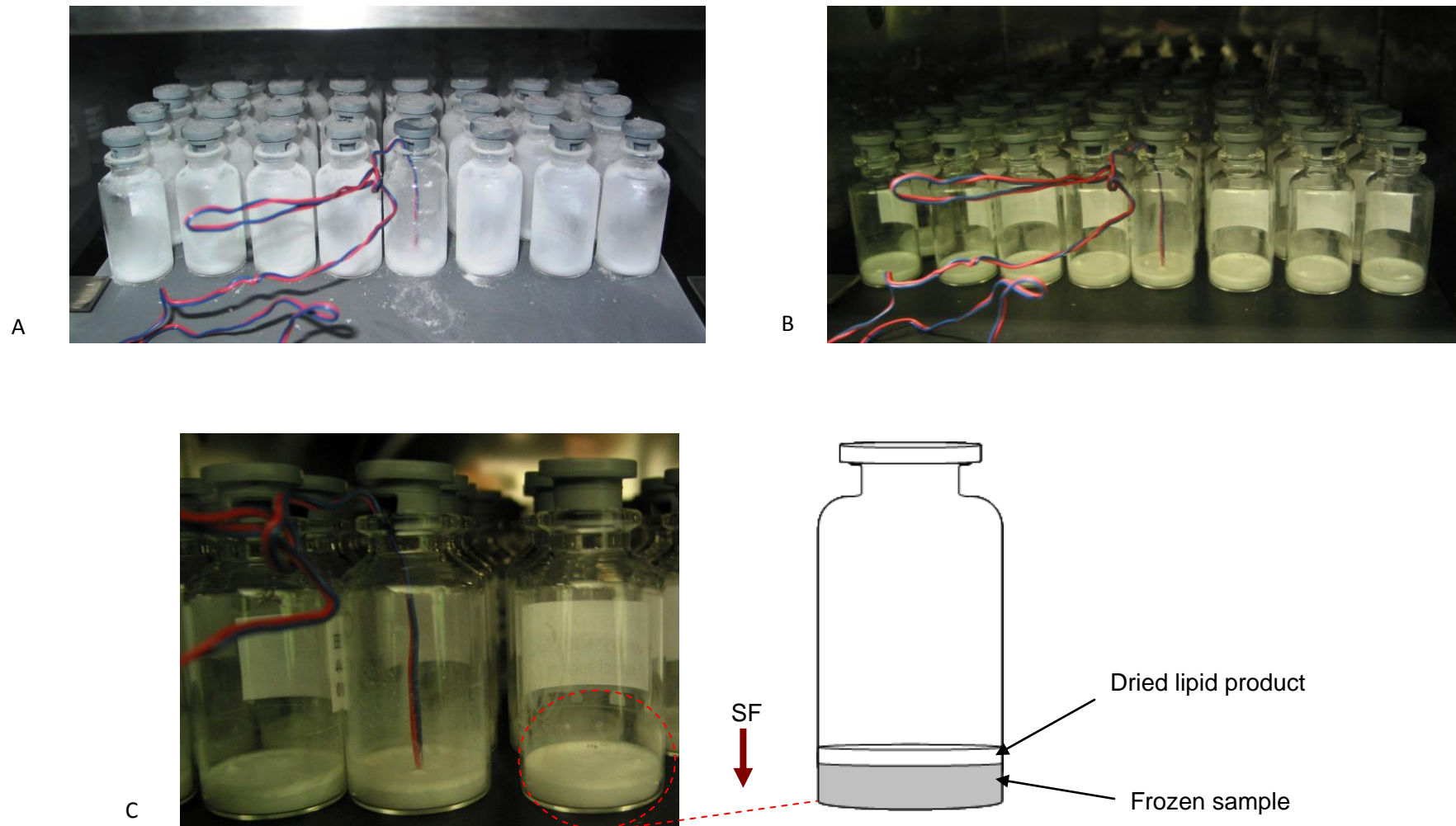


Figure 6.3: Samples undergoing lyophilisation where: **A)** Demonstrates samples removed from the -70°C pre storage conditions, **B)** Samples are in process during a freeze drying cycle. **C)** Sublimation taking place within a vial containing the niosome formulation. Temperature probes are constantly monitoring internal temperature over the time period of the lyophilisation cycle.

This was carried out as presented in figure 6.3 where thermocouples were added to vials containing niosome vesicles. Figure 6.4 presents this data where the shelf temperature was kept constant at -40°C and the change in temperature based on the thermocouples recorded the temperatures with and without a cryoprotectant. Figure 6.4 shows that there was no deviation in temperature change between 30 -75 hours of primary drying, implying that the temperature within the lipid cake is constant thus suggesting that a primary drying cycle should be chosen implementing primary drying time between this time point. Figure 6.4 also demonstrates that the addition of a cryoprotectant (200 mM sucrose) allows primary drying to take place at a much higher temperature (-20°C) as presented by the yellow line on the graph.

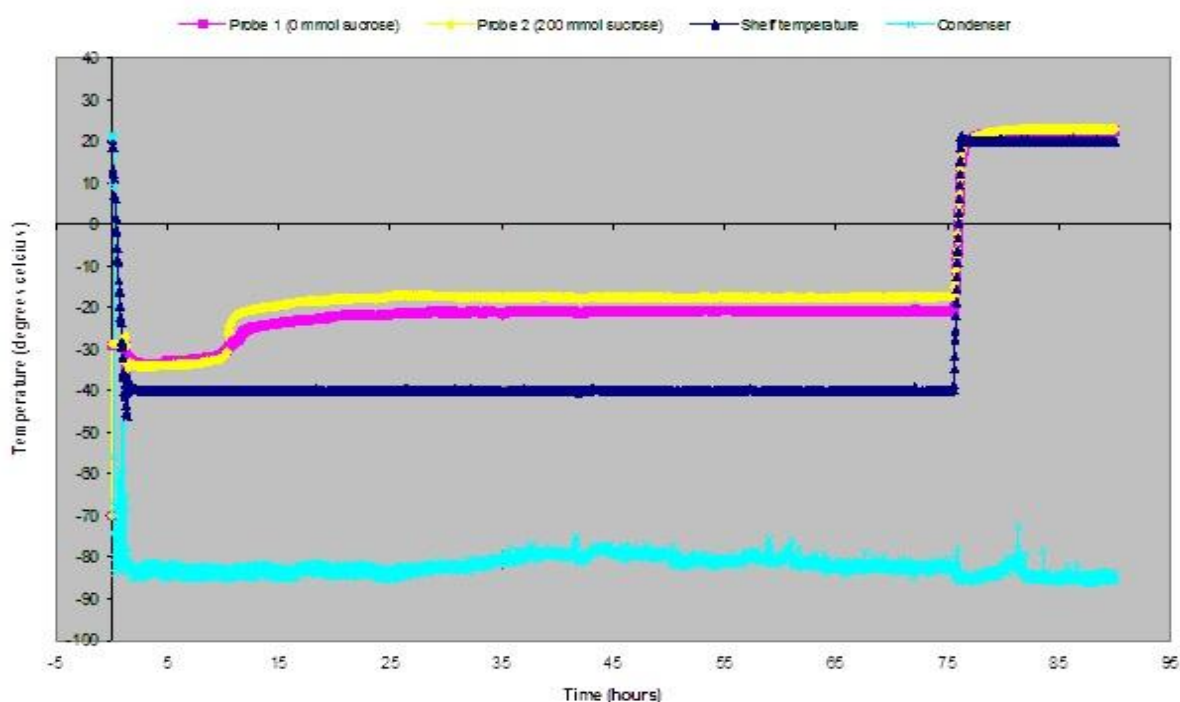


Figure 6.4: Key stages during a freeze drying cycle taken using a thermocouple placed inside niosome vials with and without sucrose.

The final optimised protocol (Table 6.2) for niosome/ bilosome vesicles was optimised for primary drying to occur for 48 hours with a shelf temperature of -40°C to ensure that the lyophilisation cycle was efficient and allowed for maximum number of vials for

large scale drying to be successfully carried out. Freeze fracture images (Figure 6.5) confirm the presence of niosome vesicles prior to and after freeze drying, where figure 6.5A represents vesicles prior to freeze drying and figure 6.5B representing rehydrated vesicles after freeze drying with the corresponding characterisation data in table 6.2B.

Table 6.2: A) Final optimised parameters for lyophilisation of niosome vesicles with condenser temperature set at -75 °C and B) Characterisation data for niosome vesicles prior to and after lyophilisation.

A	Temperature (°C)	Time (h)
Pre- freezing	-70	1h minimum
Primary drying	-40	48
Secondary drying	+20	10

B	Pre lyophilisation	After lyophilisation
Vesicle size	6.12 ± 0.09	5.85 ± 0.31
Zeta potential	-64.5 ± 6.9	-66.8 ± 9.7

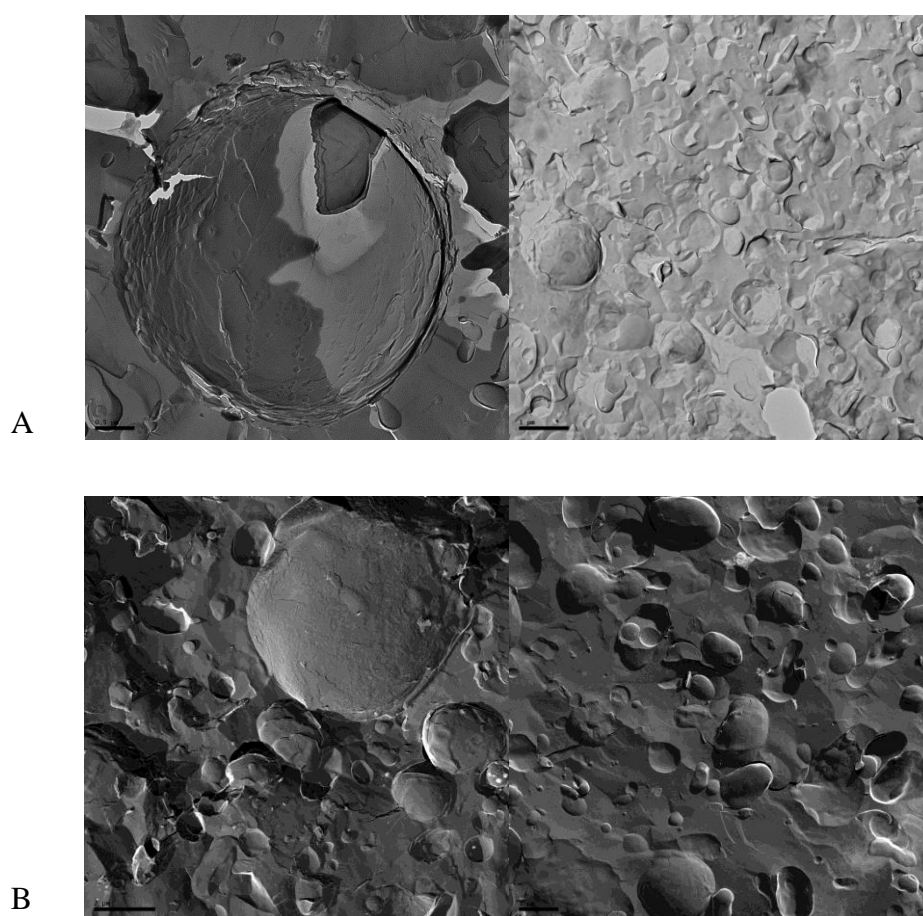


Figure 6.5: Freeze fracture images of niosome vesicles taken on a Balzers apparatus at -110 °C where images were then taken on a Jenway transmission electron microscope where: **A)** represents vesicles prior to freeze drying and **B)** rehydrated vesicles after freeze drying.

6.3.2 Oral sachet

Freeze drying a niosomal vaccine suspension results in the formation of a light powdered cake which upon rehydration results into the original liquid dosage form which can either be injected or administered orally. Freeze drying single doses within glass vials for mass production can cause an issue in terms of logistics as they occupy a large surface area and this is a less cost effective procedure. As a result, to overcome this issue, an innovative freeze dried vaccine heat sealed aluminium pouch, as presented in figure 6.6, was trialled for suitability to contain a freeze dried vaccine product. An advantage of using aluminium heat sealed pouches are that they are able to convect and conduct the 'heat' transfer process at a faster rate than if the energy was being passed through glass or plastic thus improving drying times.



Figure 6.6: Packaging options for the niosome vaccines including moisture free pouches and tablet blister packs. Images taken from Amcor and Daklapack group.

Figure 6.6 shows moisture protective heat seal or spout aluminium bags which are ideal to prevent moisture from entering the product. Niosomes were prepared in a 5:4:1 ratio of MPG:Chol:DCP respectively and after formulation mixed 1:1 with 400 mM trehalose as the protectant based on the freeze drying protocol developed at Aston university (Figure 6.4 and Table 6.2) as part of a contract research patent (Anderson et al., 2012). The pouches were filled with 20 doses and then pre frozen at -70 °C for 1 hour prior to running the freeze drying cycle. Table 6.3 shows that the vesicle size prior

to freeze drying resulted in a consistent vesicle size to all previous studies of $6.22 \pm 0.12 \mu\text{m}$ with a surface charge of $-68.5 \pm 8.6 \text{ mV}$.

Table 6.3: Characterisation data for 20 niosome vaccine doses (each dose 0.5 mL) of 5:4:1 MPG:Chol:DCP respectively demonstrating vesicle size and surface charge prior to and after freeze drying from pouches (n=3).

	Pre freeze drying	Post freeze drying
Vesicle size (μm)	6.22 ± 0.12	5.82 ± 0.25
Zeta potential (mV)	-68.5 ± 8.6	-62.3 ± 10.2

The freeze drying process resulted in a light fluffy cake being produced within the pouches which were then sealed. Upon vigorous handling of the pouches it was noticed that the cakes would crumble into a light fine powder and upon opening of the pouch a small percentage of powder would form a dust cloud and leave the pouch. Lyophilised products are subject to being poorly stable under stressed conditions in terms of physical properties such as fragility, rendering conventional packaging unsuitable (Shukla et al., 2009, Fu et al., 2004). As a result, the second pouch was carefully opened and rehydrated to see if the vesicles were still present and the vesicle size of $5.82 \pm 0.25 \mu\text{m}$ and surface charge of $-62.3 \pm 10.2 \text{ mV}$ (Table 6.3) was not significantly different to the size or surface charge prior to freeze drying.

Studies by Mohammed et al, (2006) also demonstrate that freeze drying of cationic liposomes with trehalose and subsequent sterilisation of the lyophilised cake showed no significant differences after rehydration in vesicle size, zeta potential, pH and dynamic viscosity (Mohammed et al., 2006). The vesicles remained intact as was confirmed by light microscopy of the formulations prior to and after freeze drying. The use of the cryoprotectants such as trehalose preserve functionality and activity of liposome vesicles thus increasing shelf life of the product (Mohammed et al., 2006, Zurbriggen et al., 2010). This process of having small sachets containing the vaccine which can be added to a glass of water offers several advantages to mass vaccinations and increased

patient compliance due to needle free vaccines. The setback to this preparation method is that due to the freeze drying products being of a light flaky powdered nature this could result in incorrect dosing issues as there is a chance of the vaccine being lost upon opening of the sachet. Therefore, an alternative solid dosage form was investigated.

6.3.3 Development of a rapid disintegrating tablet

As previously shown (Section 6.3.2), the niosome vesicles are able to undergo the freeze drying process with a protectant and no significant changes in vesicle size or surface charge were noted upon rehydration. The underlying problem to the sachet was the nature of the final product was very delicate and needed to be handled with care to not lose any dose. As a result, a more robust final product is required which is able to resist handling and mechanical stresses. Therefore as an alternative option, this section involved the development of a freeze-dried tablet formulation using mannitol as an additive which has known to have bulking and stabilisation properties on the final freeze dried product (Seager, 1998). Mannitol is also water soluble and non-hygroscopic which provides a cooling sensation when in the mouth (Jeong et al., 2008). In addition, sucrose will be used as a comparison with trehalose to determine any effects of protectant on the final product characteristics in table 6.4. The cryoprotectant was initially prepared at a 200 mM concentration which resulted in a final concentration of the sample and cryoprotectant being 100 mM.

Initial indications (Figure 6.7) showed that two out of five samples for the niosomes protected with sucrose collapsed during freeze drying (Figure 6.7 vials 2C and 2D). The other three sucrose samples and the trehalose formulations all formed nice tablet shaped cakes. The collapsing of the niosomes was a result of a low final concentration of cryoprotectant which should have started off at 400 mM resulting in a final

concentration of 200 mM. Future studies rectified this issue and a repeat study showed the use of 200 mM final concentration resulted in no collapse of the tablet cakes. Redispersed of the samples (Table 6.4) was carried out with 2 mL water into the vials and gently agitated where the resuspension time was less than 5 seconds. The dispersion time of the tablets was rapid due to the matrix being prepared from water soluble sugars (sucrose/trehalose) thus, rendering the moulded tablets to rapidly disintegrate and offer improved taste (mannitol). The presence of mannitol to the tablets offers high aqueous solubility and sweetness, offering taste masking resulting in a pleasant feel within the mouth (Jeong et al., 2008, Mizumoto et al., 1996). However, these moulded tablets resulted in poor mechanical strength where figure 6.6 demonstrated that removal of the tablets from the moulds, handling was compromised.

Table 6.4: Niosome formulations and pre characterisation data. (5:4:1 niosomes of MPG:Chol:DCP respectively, with protectant final concentration 100 mM)

Niosome	Additives	Protectant	Pre Freeze drying		After Freeze drying	
			Vesicle Size (μm)	Zeta potential (mV)	Vesicle Size (μm)	Zeta potential (mV)
1	5% w/v	Trehalose	5.88 ± 0.05	-68.54 ± 6.4	6.55 ± 0.12	-59.8 ± 8.9
	Mannitol					
2	5% w/v	Sucrose	5.88 ± 0.05	-68.54 ± 6.4	8.42 ± 0.45	-54.8 ± 12.1
	Mannitol					
3	N/A	Trehalose	5.65 ± 0.08	-70.82 ± 6.4	6.45 ± 0.22	-65.7 ± 4.8

These initial freeze drying techniques showed that tablet shaped cakes (Figure 6.7) were formed which were able to redisperse back into vesicles upon the addition of an aqueous phase. Table 6.4 shows that the addition of sucrose as the cryoprotectant resulted in a significant ($p < 0.05$) increase in vesicle size after rehydration compared to trehalose formulations with no significant difference in zeta potential. The underlying problem with the sucrose and trehalose tablets within this preliminary study was the low final concentration of protectant within the formulations and hence required double the concentration. In addition, for a more robust tablet ie. a very hard lyophilised cake

the addition of a binder to the formulations which does not interact with the vesicles to change their morphology was examined based on the excipients listed in table 6.1. This formed the basis of the next studies which introduced the use of a binder such as dextran or gelatin to the niosome formulations prior to lyophilisation to provide structural integrity of the tablets.

In general, freeze dried tablets such as Claritin[®] RediTabs[®] have highly porous inner structures which results in immediate disintegration and dissolving of the tablets upon the tongue (Fu et al., 2004). The highly porous nature of the freeze dried tablets results in their fragile properties. To increase, handling of the tablets, either binders or other excipients can be used or the preparation method can be altered. In terms of inducing hardness, Benadryl[®] Fastmelt[™], using the WOWTAB[®] technology (Pfizer) reduce tablet porosity by direct compression of the tablets resulting in harder tablets (Fu et al., 2004, Dobetti, 2000).

6.3.4 Introduction of a Binder

Formulations prepared previously (Section 6.3.3) had shown the ability to form a nice cake (Figure 6.7); however, structural integrity was lacking and handling was a fundamental weakness. As a result a suitable agent to increase hardness and manual handling of the tablets was investigated. The tablets were prepared based on table 6.3 and after lyophilisation were tested for hardness, disintegration time and upon rehydration, characterised for vesicle size and zeta potential. To increase the hardness of the tablets, excipients including dextran and gelatin were chosen (Table 6.3).

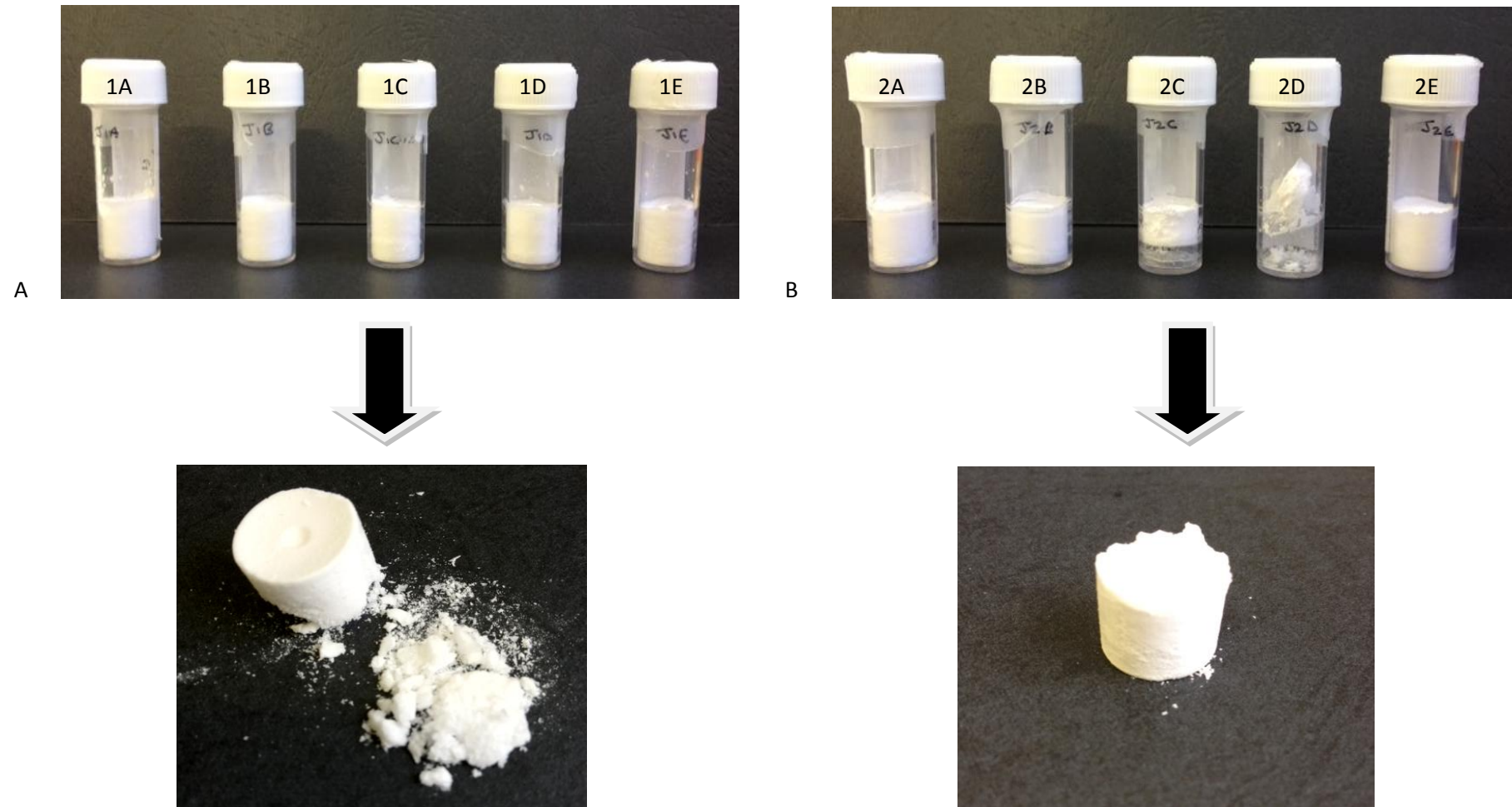


Figure 6.7: Final lyophilised tablets of niosomes (5:4:1 of MPG:Chol:DCP respectively) containing 5% w/w mannitol representing appearance and handling protected with 100 mM final concentration of either **A)** trehalose and **B)** sucrose (n=5).

Results show that the presence of mannitol caused in a slight reduction although not significant of the zeta potential (Table 6.6) which was most likely due to its presence around the stern layer when measuring the zeta potential. This was also demonstrated by Budai et al, (2009) where the addition of glucose and mannitol to liposome vesicles was shown to possibly mask the liposomal vesicle charge (Budai et al., 2009). In addition, the presence of salt within liposomal formulations influences the surface (Moghaddam et al., 2011b). Upon rehydration the dextran and mannitol formulations appeared to have no significant difference in terms of vesicle size and zeta potential and showed that after rehydration the vesicles returned to their original characteristics (Figure 6.8A). When using the gelatin excipient within the formulations, it was only soluble in water at elevated temperatures where the gelatin had to be pre-heated at 45 °C and left to dissolve. An aliquot of this solution was then added to the vesicle formulation. When working with gelatin it was noted that upon cooling back down to room temperature the gelatin was prone to precipitating back out of solution and becoming viscous prior to use, thus potentially causing problems with temperature sensitive vaccine antigens. Furthermore, the incorporation of gelatin to the niosome formulations after freeze drying, resulted in a significant reduction ($p < 0.05$) in vesicle size ($< 4 \mu\text{m}$) upon rehydration (Figure 6.8A).

Table 6.5: Formulation design study including the use of dextran and gelatin in combination with mannitol added to 5:4:1 niosomes of MPG:Chol:DCP respectively (n=3).

Niosome formulation	Additives	Cryoprotectant final concentration
4	Mannitol 5 % w/v, Dextran 10 % w/v	200 mM Trehalose
5	Dextran 10 % w/v	200 mM Trehalose
6	Gelatin Final Conc 0.5 % w/v	200 mM Trehalose
7	Gelatin 0.5 % w/v, Mannitol 5 % w/v	200 mM Trehalose

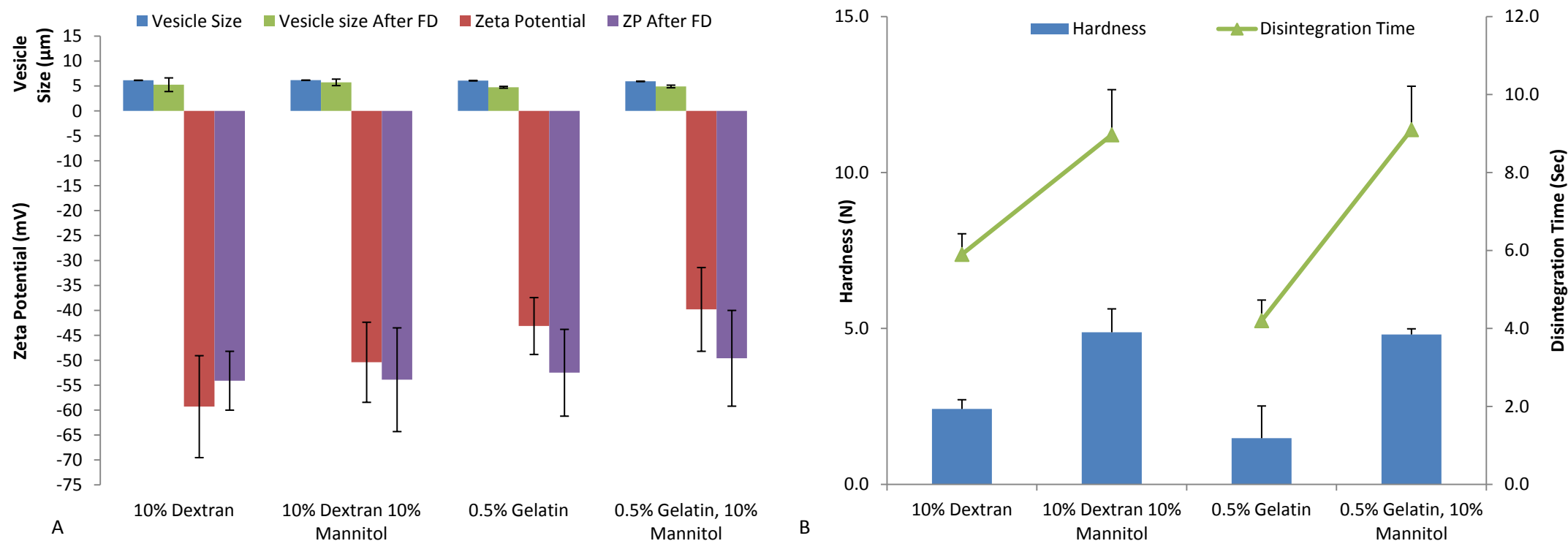


Figure 6.8: The impact of dextran and gelatin in combination with mannitol added to preformed niosome vesicles on: **A)** vesicle size and zeta potential pre and after freeze drying and **B)** the hardness and disintegration times of the tablets (n=5).

In addition, the inclusion of mannitol within the formulations after freeze drying resulted in harder tablets (Figure 6.8B), represented by the hardness results which were carried out by a Hounsfield machine which applied a force 2 mm into the tablet where resistance to indentation produced the hardness result. The addition of mannitol has previously shown to induce crystallinity within the lyophilised cake thus imparting rigidity and preventing collapse of the structure (Sastry et al., 2000). Studies have shown that the matrix of freeze dried tablets that consist of polymers such as gelatins, dextrans or alginates provide structural strength and saccharides such as mannitol or sorbitol provide crystallinity, hardness and elegance (Sastry et al., 2000). This trend is apparent in the dextran and gelatin formulations, where the presence of mannitol has significantly ($p < 0.05$) improved hardness of the tablets (Figure 6.8B).

In terms of disintegration time, the inclusion of mannitol significantly ($p < 0.05$) increased the time of the tablets to disintegrate from 4-6 seconds to 9 seconds which was related to the increased hardness of the tablets thus requiring a longer period of time to disintegrate (Figure 6.9B). All tablets produced within this study are categorised within the rapid disintegrating tablets class based on the criteria by the FDA where rapid disintegrating tablets should disintegrate in less than 30 seconds (McLaughlin, 2009, FDA, 2008). After assessing the tablets produced within this study the dextran and mannitol based combinations formed the basis of the next studies with the aim to improve hardness by determining optimum concentrations of the excipients. The gelatin formulations were discarded due to the inclusion of extra preparation steps which included heating the gelatin to 45 °C as this may have an impact when using thermolabile antigens. In addition, aesthetically the gelatin based tablets were not appealing due to an off white / yellow discolouration due to the presence of gelatin.

6.3.5 Modifications to oral tablets

Formulations prepared in section 6.3.4 resulted in the tablets with dextran and mannitol providing suitable candidates for a vaccine tablet. As a result, different combinations of dextran and mannitol were prepared with the inclusion of a strawberry flavouring to one of the formulations (Table 6.6). The formulations prepared were once again tested for tablet hardness, resuspension to confirm vesicle size and zeta potential and then thermogravimetric analysis (TGA) studies to determine moisture content levels within the final freeze dried products. Freeze dried products are required to contain less than 5 % moisture content as the maximum moisture content within the final freeze dried product.

Results within table 6.6 show that prior to addition of excipients the batches of vesicles produced are very reproducible showing a consistent vesicle size and zeta potential. Upon addition of excipients the vesicle size range decreases which could be due to the dextran pulling the VMD lower as the smaller vesicle size range increases. The addition of 5 % dextran results in the VMD remaining consistent with the initial VMD (Table 6.6). Once again this study confirms that the reduction in zeta potential occurs due to the presence of mannitol within the formulation as prior to mannitol addition the zeta potential is in the -64 mV range. After lyophilisation, rehydration of the samples show that the vesicle size (Table 6.6) for all of the formulations was approximately 4.5 μm which showed uniformity between the various formulations after freeze drying. The zeta potential values upon rehydration showed no significant differences between them and are all lower than the initial readings prior to excipient addition as presented in Table 6.6.

Dextran and mannitol, are key excipients in controlling mechanical hardness and disintegration time (Chandrasekhar et al., 2009, Seager, 1998). Figure 6.9 demonstrates

the tablet hardness procedure for the 10 % dextran, 10 % mannitol and 1 % strawberry niosome tablets showing resultant indentation and fracturing of the tablet. Figure 6.10B shows that the increase in mannitol concentration to 15 % w/v of the formulations, resulted in fracturing of the tablets compared to formulations with only 10 % w/v. The addition of 1 % v/v strawberry flavour to the formulation showed significantly ($p < 0.05$) higher tablet hardness (16 ± 1.4 N) where the tablets were prone to fracturing, resulting in the longest disintegration time. This could be due to the adhesive properties of the flavour which has helped the formulation adhere together hence resulting in increased disintegration and hardness.

Table 6.6: Formulation specifications showing characterisation data for niosomes (5:4:1 MPG:Chol:DCP respectively) for vesicle size and zeta potential at key stages including: Formulation of initial vesicles, after addition of excipients prior to FD and upon rehydration after freeze drying (n=5).

	10% Dextran, 10% Mannitol, 1% Strawberry	5% Dextran, 10% Mannitol	10% Dextran, 15% Mannitol	10% Dextran, 10% Mannitol	5% Dextran, 15% Mannitol
Initial Vesicle size (μm)	6.12 ± 0.05	6.31 ± 0.22	6.03 ± 0.18	6.08 ± 0.02	6.18 ± 0.10
Initial zeta potential (mV)	-60.9 ± 4.5	-63.6 ± 2.8	-63.6 ± 8.9	-68.5 ± 2.7	-67.0 ± 10.2
Vesicle size with excipients (μm)	3.93 ± 0.45	6.19 ± 0.23	4.90 ± 0.65	5.71 ± 0.33	6.14 ± 0.12
Zeta potential with excipients (mV)	-53.2 ± 10.8	-50.2 ± 6.1	-60.0 ± 8.6	-51.6 ± 7.3	-53.2 ± 6.6
Vesicle size after FD (μm)	4.25 ± 0.33	4.67 ± 0.56	4.69 ± 0.12	3.98 ± 0.22	4.86 ± 0.11
Zeta potential after FD (mV)	-45.4 ± 3.6	-52.8 ± 6.5	-49.2 ± 7.3	-53.5 ± 7.3	-53.9 ± 7.5



Figure 6.9: Formulation 8 containing 1 % strawberry, 10 % dextran and 10 % mannitol (very elegant aesthetics) undergoing a hardness test using a 5 mm diameter probe showing indentation with fracturing.

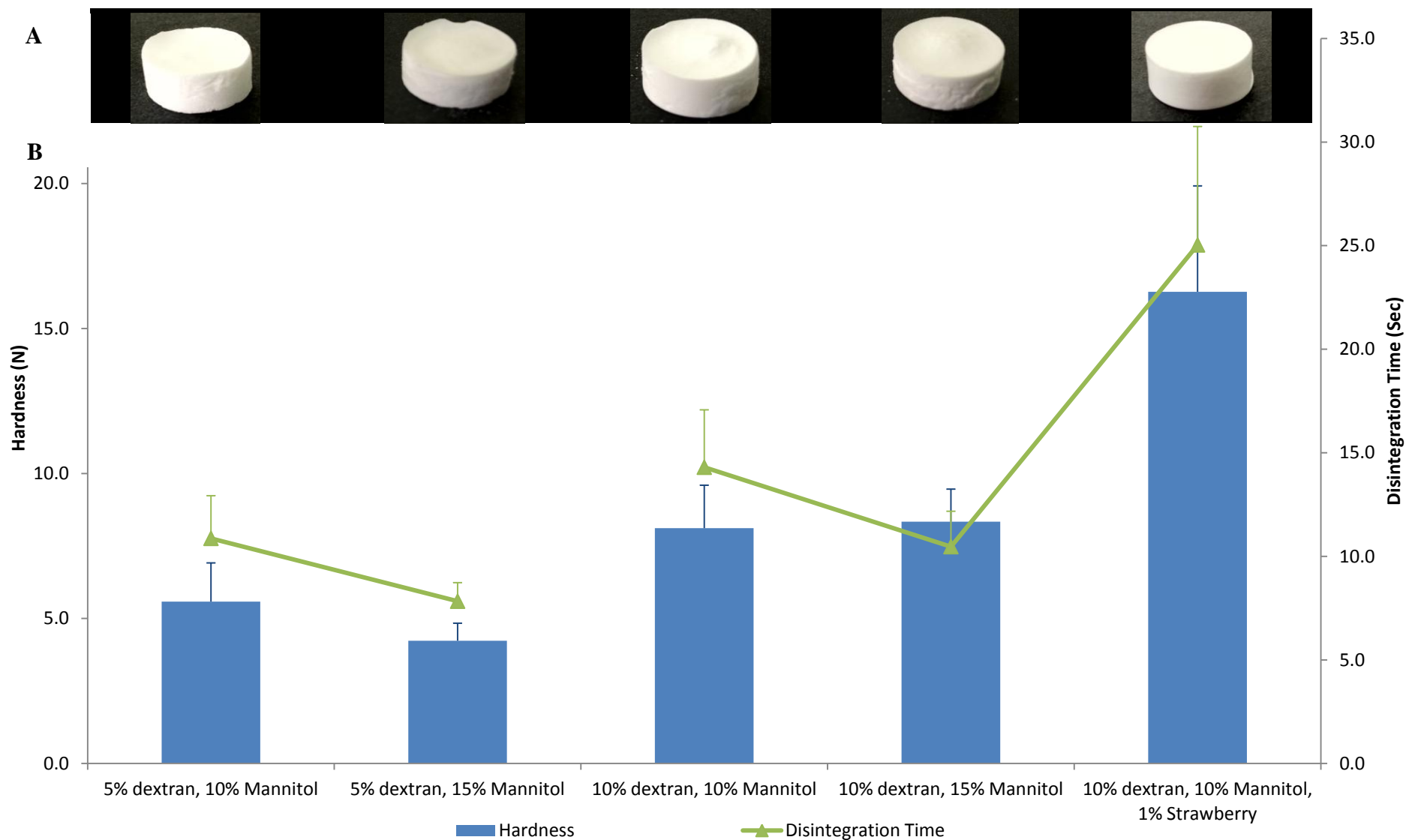


Figure 6.10: The result of varying mannitol and dextran ratios within the niosome mixture where; **A)** presents the appearance of the tablets and **B)** the impact on tablet hardness and disintegration time (n=5).

Based on the current freeze drying studies, it is possible to prepare a tablet dosage form with structural integrity. The pharmacological importance of moisture present within the lyophilised tablet is the sensitivity of the antigen/excipients to residual moisture, which can impair the medicinal effect of the tablet and can impair long term storage. This fact is verified by the specifications in the British Pharmacopeia, which mandates that tests for microbial contamination must be performed if moisture is present (> 5 %) in solid dosage forms. Moisture harbours the growth and reproduction of microbes which provide optimum growth conditions for the bacteria which is unacceptable in oral dosage forms. Hence, it is essential to make sure that the final product post lyophilisation is free from excess moisture and so does not cause contamination issues. Furthermore, traces of moisture in a tablet may lead to longer term stability issues such as, friability, dissolution and loss of potency of the drug (which may eventually change the permeability of any coatings applied and interfere with the performance of the coating layer) (Sugimoto et al., 2006, Nakabayashi et al., 1980). In addition to manufacturing methods it is crucial that the packaging for storage and handling of the tablets are not sensitive to moisture and provide an effective barrier for moisture (Figure 6.1). To determine moisture content within the formulations, thermogravimetric analysis studies (Table 6.7) were carried out (Figure 6.11).

Table 6.7: Moisture content for all batches of tablets (n=3) (5:4:1 niosomes of MPG:Chol:DCP respectively).

Formulation	Moisture Content (%)
10% Dextran, 10% Mannitol, 1% Strawberry	4.1 ± 0.1
5% Dextran, 10% Mannitol	3.8 ± 0.3
10% Dextran, 15% Mannitol	3.1 ± 0.1
10% Dextran, 10% Mannitol	2.6 ± 0.2
5% Dextran, 15% Mannitol	3.1 ± 0.6

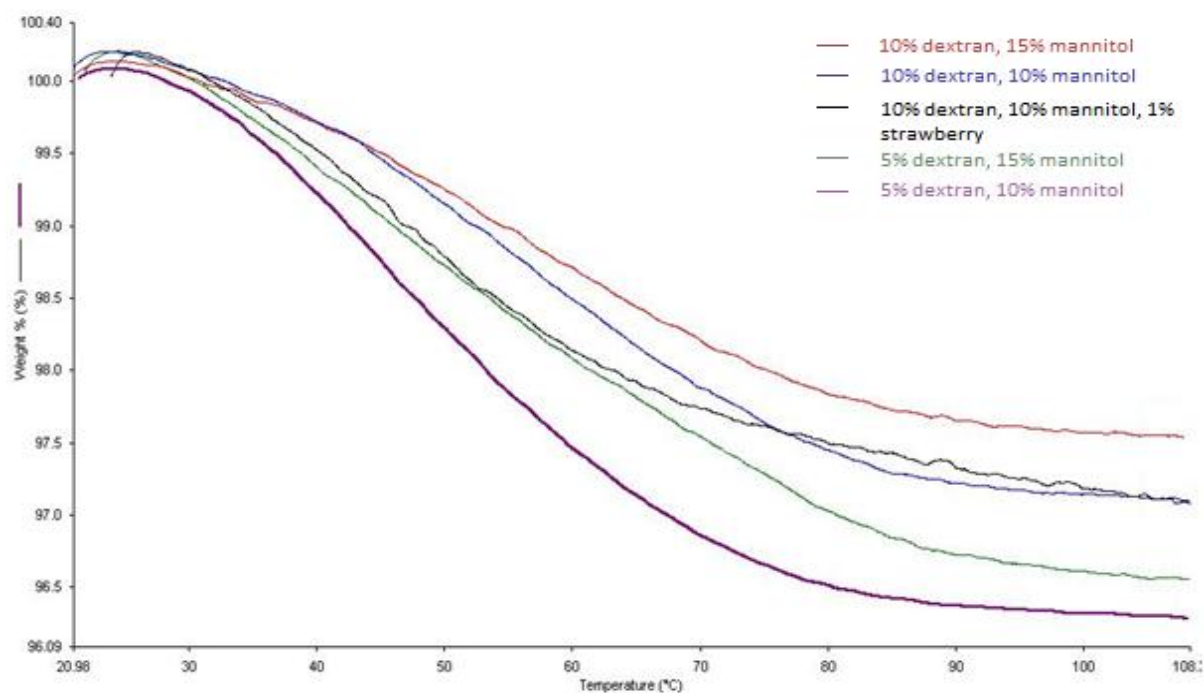


Figure 6.11: TGA scans overlaid for all formulations showing % moisture content where each formulation was carried out (n=3).

Moisture content analysis was carried out using TGA based on a similar protocol to Mohammed et al, (2006) (Figure 6.11). The limits for a freeze dried formulation are that no tablet should contain more than 5 % moisture content and the thermogram scans (Figure 6.11) and data (Table 6.7) show that all of the tablets prepared pass the moisture content test. The moisture content results within this study are in line with Mohammed et al, (2006) where they showed that cationic liposome vesicles with the use of trehalose as the cryoprotectant after freeze drying showed residual moisture content levels to be less than 5 % (Mohammed et al., 2006). The freeze drying method employed a secondary drying phase at +20 °C for 10 hours. However, TGA scans (Figure 6.11) show that additional moisture content can be eliminated from the tablets by holding the secondary drying temperature at a higher temperature like 50 °C as well as the +20 °C, potentially removing another 1.5 % moisture from the tablets. This extra moisture thermal treatment if necessary can significantly improve the mechanical strength of tablets due to the removal of moisture bridges within the product (Fu et al., 2004). As the tablets have passed the requirements, there would be no need to add this

extra step as this would cost time, money and expose any thermolabile antigens or drugs to the elevated temperature. When correlating the data together, moisture content analysis was compared to tablet hardness (Figure 6.12). The results show that dextran content of 5 % w/v results in higher moisture content (~4 %) within the tablets and also results in the reduced hardness (4-6 N) of the tablets (Figure 6.12). Studies by Nakabayashi et al, (1980) also demonstrated that the increase in moisture content within the lactose- cornstarch tablets rapidly decreased the hardness of the tablets (Nakabayashi et al., 1980). In addition, the tablets containing the higher residual moisture were not prone to fracturing; however, did leave an indentation after the hardness test was applied (results not shown). The low moisture containing tablets (< 3.5 %) resulted in the tablet fracturing into 3-4 pieces upon force suggesting that the tablets are drier and hence are more inclined to fracture. Studies by Sugimoto et al, (2006) demonstrate that mannitol absorbed little moisture upon increase in relative humidity compared to excipients such as glucose or sorbitol which absorb more moisture upon increases in relative humidity (Sugimoto et al., 2006). As previously shown in section 6.3.4 the presence of mannitol within the formulations resulted in an increase in tablet hardness. Furthermore, the increase in moisture content within the tablets results in decreased tensile strength upon storage, which reflects our studies (Figure 6.12) where (although not significant) the increased moisture content tended to result in lower hardness tablets (Sugimoto et al., 2006). The presence of water has shown to act as a plasticiser during freeze drying, whereby water can affect the physical properties of the freeze dried solid by inducing cake softening, cake collapse, crystallisation of amorphous solids, polymorphic conversions between crystalline structures and protein aggregation by increasing mobility within the solid (Costantino and Pikal, 2004).

Figure 6.13 shows the residual moisture content of the formulations and is compared to the disintegration times of the tablets. The results show a similar trend for both responses although not significant however, the higher the moisture content within the tablets the faster the disintegration time (Formulation 9 and 12). This is attributed to the tablets containing higher residual moisture content showing parts of the tablet are slightly wet and are more cohesive and upon rehydration disintegrate faster, whereas the drier tablets require water to be taken up and then disintegration to occur.

Disintegration occurs faster with the tablets which contain the low dextran content of 5 % where the moisture content of those tablets was higher and thus, resulted in faster disintegration times. In addition, increased mannitol concentrations within tablets has been shown in literature to increase the disintegration and dissolution rate of tablets (Chandrasekhar et al., 2009, Mizumoto et al., 1996, AlHusban et al., 2010). Chandrasekhar et al, (2009) demonstrated that the increase in mannitol within tablet formulations resulted in increased dissolution rates which was due to the high water solubility of the mannitol (Chandrasekhar et al., 2009). Hence, when increasing the mannitol concentration, it is important to increase the dextran content as this will increase tablet hardness and in turn increasing the collapse temperature of the formulations thus increasing the stability of the product at elevated temperatures.

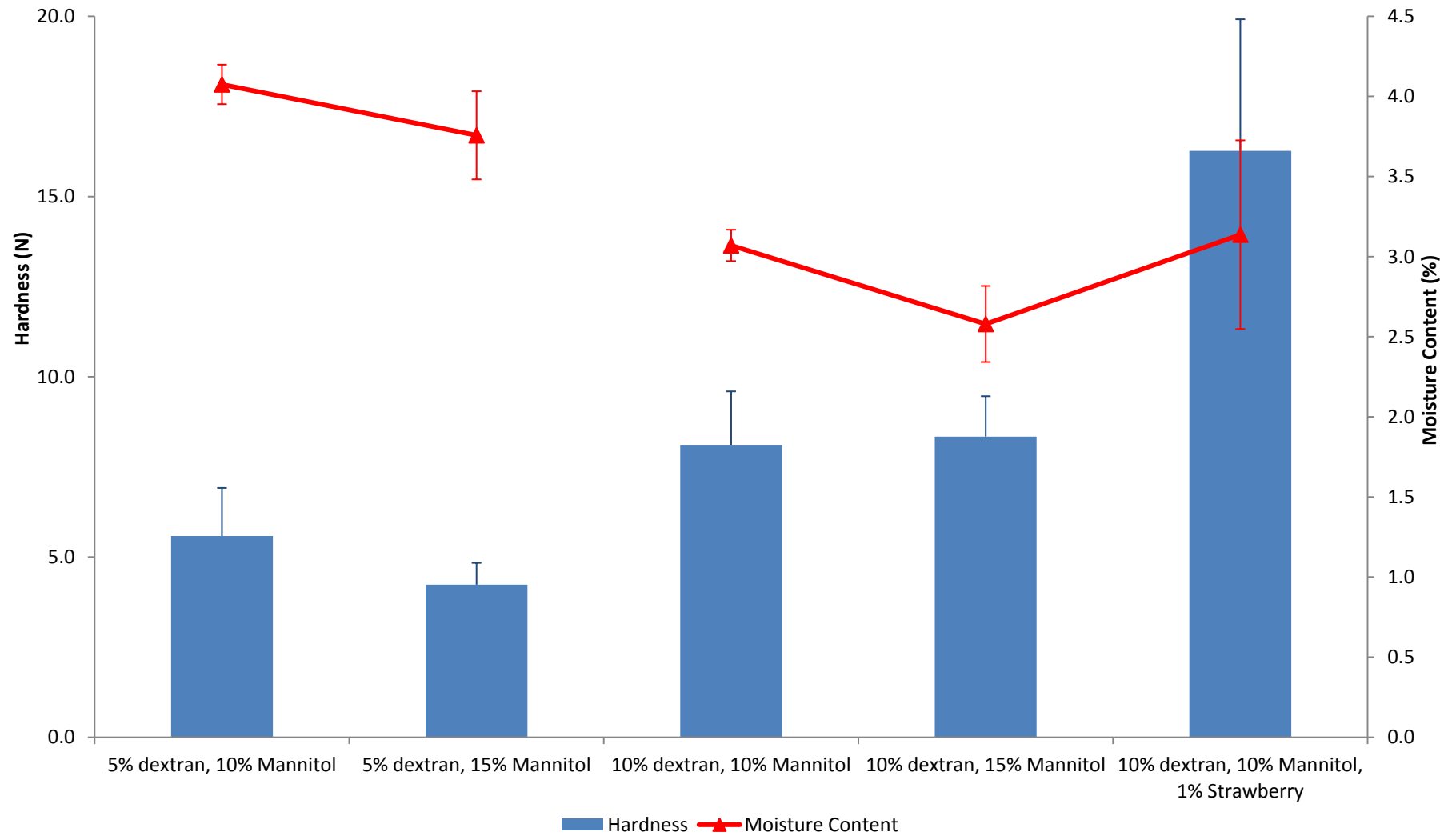


Figure 6.12: The effect of moisture content on the hardness of the freeze dried tablets at different concentrations of dextran and mannitol (n=5).

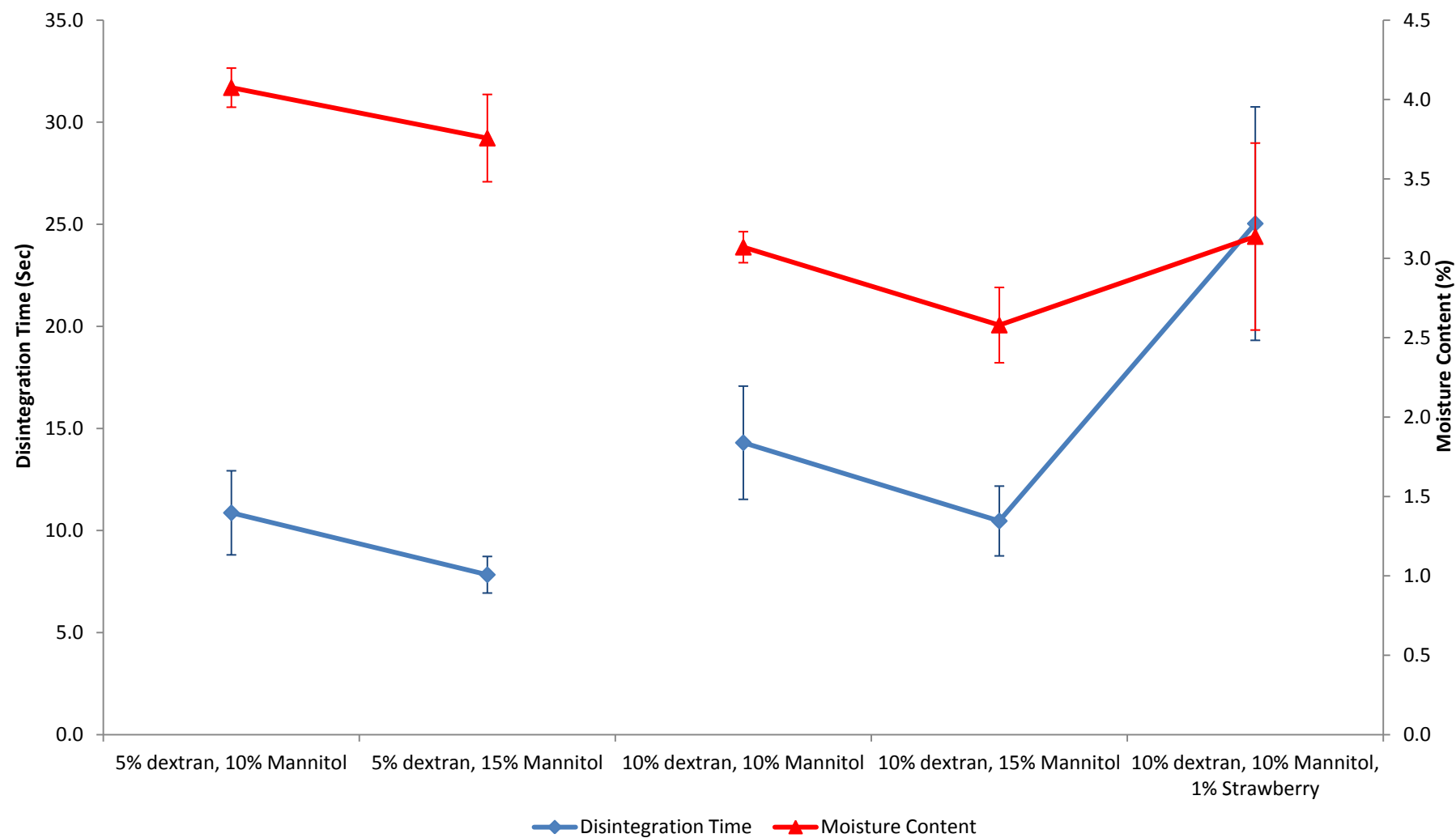


Figure 6.13: The effect of moisture content of the tablets after freeze drying on the disintegration time of the tablets at 37 °C (n=5).

6.4 Formulation of a vaccine capsule

As previously shown within this chapter (section 6.3), results show that prior to adding mannitol and dextran to the niosome vesicles the average VMD of the vesicles is approximately 6 μm and upon addition of excipients decreases to 4-6 μm which is a result of the excipients bringing the VMD down and not so much the vesicles reducing in size. Upon rehydration after freeze drying the vesicle size averaged out to 4-6 μm with the zeta potential remaining unchanged. Moreover, the vaccine tablets produced were classed as rapid disintegrating tablets or oral chewable tablets which have several benefits. However, the tablets produced (section 6.3) still expose the vesicles and vaccine to the external milieu of the stomach which resulted in niosome vesicles losing antigen when exposed to gastric conditions as demonstrated in chapter 5. As a result, due to the moisture sensitivity of the freeze drying tablets, enteric coating techniques all require the use of aqueous phases thus poses deterioration of the tablets during the coating. Hence, as an alternative to tableting, freeze dried vaccine formulations within capsules were examined.

Thus, the aim of this study was to prepare niosome vaccine formulations containing H3N2 antigen to load within capsule shells and compare the rehydration characteristics to a control tablet to ensure the capsule is not interfering with the vesicles. The intention of the capsules was to reduce contact time of the stomach acids and enzymes to the vaccine formulation thus offering increased stability within the GIT.

6.4.1 Niosomes for oral delivery via capsule dosing

Empty niosomes were produced at a 5:4:1 ratio of MPG:Chol:DCP respectively mixed at a 1:1 ratio with 400 mM trehalose and 500 μL aliquots were placed into the bottom of the capsule shells and immediately frozen at -70 $^{\circ}\text{C}$. The capsules were compared to a control formulation which was freeze dried in the form of a tablet. The capsule

formulations did not require the use of additives such as dextran or mannitol as the capsule shell provides structural integrity thus increasing handling and protection of the niosome vaccines enclosed. The capsule shells are presented in figure 6.14 with a fill volume of 0.5 mL and were added to the capsule holder ensuring the bottom of the shells are able to be in full contact with the shelf for efficient lyophilisation conduction.



Figure 6.14: Empty capsule shell and one filled with the niosome wet formulation showing no leakage of the enclosed niosomes.



Figure 6.15: Capsule holder designed to hold capsule shell bases allowing the bottom of the shells to be in full contact with the lyophiliser base plate to allow efficient drying of the niosomes.

Firstly, vesicle size distribution and surface charge was analysed prior to and after the freeze drying cycle has taken place and this data is represented in figure 6.16A. The initial vesicle size is comparable to all formulations within section 6.3 with an average

vesicle size of 6-6.5 μm and a highly negative zeta potential ($> -65\text{ mV}$). After the freeze drying cycle, the capsule shell was taken out of the holder and then initially broken up to release the material to confirm if a powdered cake was formed (Figure 6.16B). Upon confirmation that the material had successfully freeze dried, the capsules were rehydrated with 0.5 mL ultrapure water, vortexed and then analysed for vesicle size and charge. The vesicles from the capsules reduced in size to an average of 4.5 μm which is in line with the control and comparable to previous data (Section 6.3) with the zeta potential remaining highly negative (Figure 6.16A) which are still attributed to uptake by the Peyer's patches (Tabata and Ikada, 1988, Ebel, 1990, Eldridge et al., 1990, Tabata et al., 1996). This data is encouraging as it shows that the niosomes are the same after freeze drying whether formed as a tablet or encapsulated within capsules.

After removing the capsules from the capsule holder it was noted that the shells had initially shrunk in size which could be due to the vacuum pressure and sublimation of water during the primary freeze drying process. This however, proved to be advantageous for the niosomal formulations, as the initial shell can be pushed into a new capsule shell and a lid placed on top to seal the capsule as shown in figure 6.16B. This therefore providing a dual barrier to the capsule protecting the niosomes from potential acid attack when in gastric conditions. Another advantage of this capsule within a capsule offers the potential to add in other excipient doses into the outer shell which will be released prior to the niosome release. This dual dosing technique within a single capsule can allow the presence of a small dose of bile acid sequestrants such as cholestyramine within the outer capsule shell which when released into the small intestine binds the bile acids and interrupts their circulation (Marounek et al., 2010, Stedronsky, 1994).

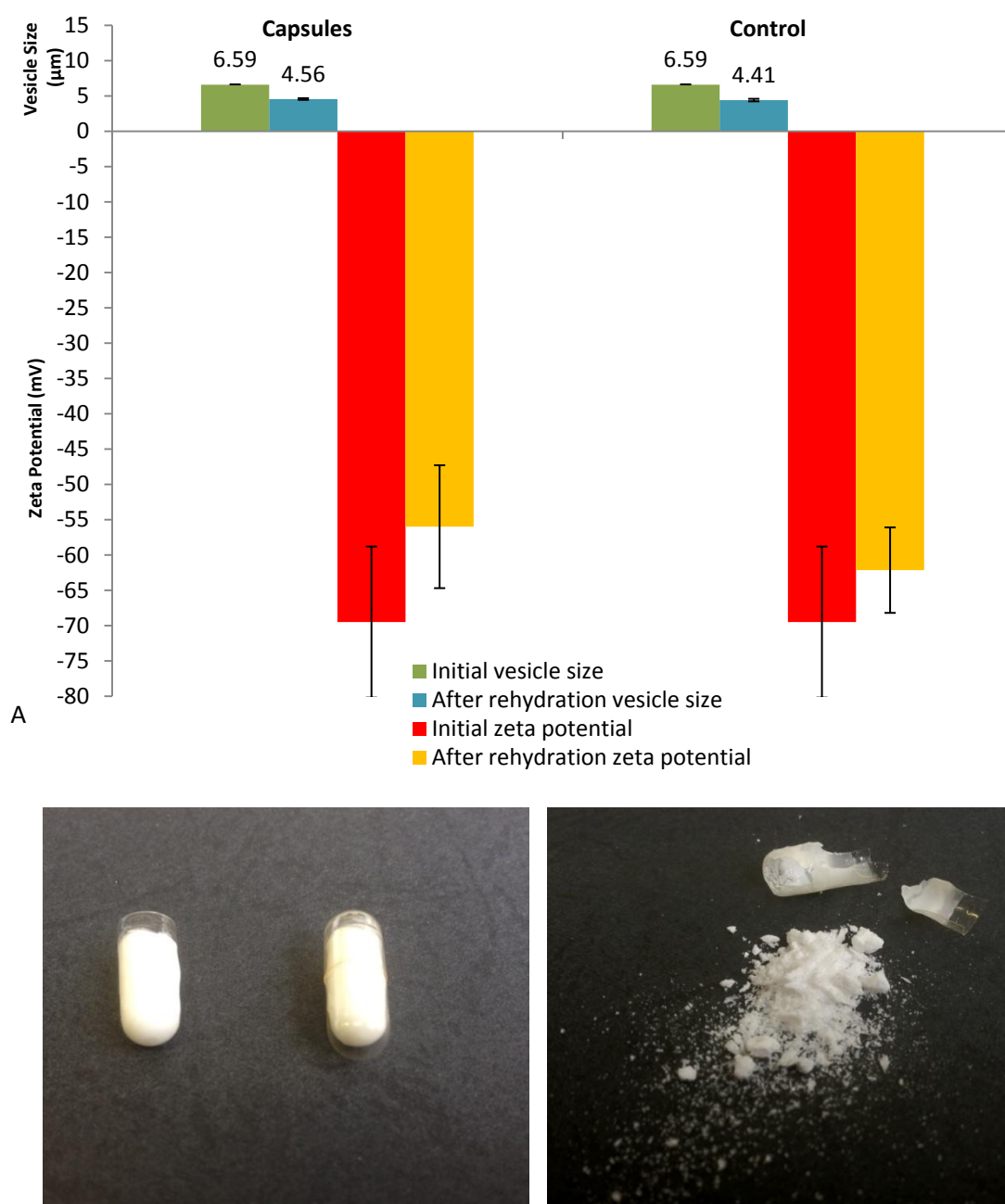


Figure 6.16: A) Vesicle size and zeta potential of the niosomes pre and after freeze drying of capsules including the tablet control (n=6) and **B)** appearance of the freeze dried capsule niosome formulation.

The removal of excess bile acid from the small intestine would prevent the bile acids from attacking the vesicles thus offering increased antigen protection with a chance of increasing the vesicle and antigen concentration within the Peyer's patches. The resultant dual shelled capsules were tested for disintegration time and moisture content as presented in figure 6.17.

Figure 6.17 represents the moisture content and disintegration time of the capsule and the control tablet formulation within gastric media (pH 1.2). Results show that the control tablets disintegrate within 5 seconds, compared to the capsules which have a disintegration/ release time of 5-6 minutes. Capsules prepared by Jones et al, (2012), have shown that tests in humans show that within the fasted state, gelatin based capsules disintegrate within 7 ± 3 min compared to a fed state of 12 ± 3 min (Jones et al., 2012). The capsules prepared within this study offer this delayed release profile of 6 minutes and is beneficial as it reduces the contact time of the niosomes and potentially any drugs / antigens prone to acid or enzymatic degradation within the stomach. In terms of moisture content, the capsules offer similar moisture content levels to the tablets previously (Section 6.3) formed and the control formulation within this experiment. The moisture content remained below 5 % thus should be sufficient to provide stability during storage. Thus, by formulating the niosomes within the capsules, they offer a promising platform to oral drug/vaccine delivery.

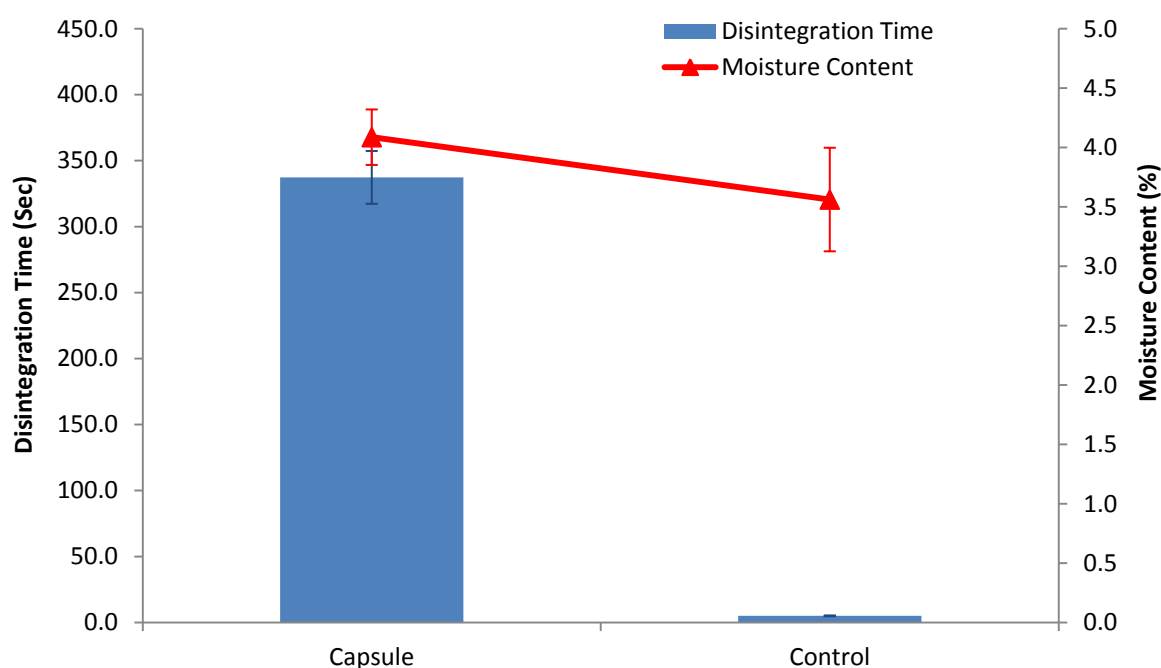


Figure 6.17: Moisture content and disintegration time (37 °C) of the capsules and control niosome formulations after freeze drying (n=5).

In summary, empty vesicles have shown to be evident and intact pre and after freeze drying and offers an alternative to liquid dosage forms. Niosomes whether in tablet or capsule form have both shown low levels of moisture content with the current freeze drying cycle used and the desired disintegration time of the formulation whether is it a rapid fast melt, oral chewable tablet or a slow release capsule. To determine whether this platform had the potential to retain antigen fluorescently labelled H3N2 antigen was associated with the vesicles to determine whether the antigen loaded into niosomes was present after the freeze drying procedure for the various dosage forms.

6.5 Determination of antigen content after freeze drying

Within this study, the aim was to prove that the antigen is present within the formulations after the freeze drying process has been carried out. Thus, the test formulations for this experiment included a niosome control formulation, a rapid fast melting tablet, oral chewable tablet and a capsule (section 6.3 and 6.4).

When determining the fluorescence of the antigen present within the niosome formulations, it was important to run a control of blank niosomes and then subtract this from the overall readings to determine actual antigen concentration without any background readings. As a result, the formulations were rehydrated and centrifuged using a two wash cycle to remove any un-entrapped fluorescent antigen within the vesicles. The samples were washed and the pellet hydrated and 100 μ L of the pellet was placed into the black microplates for fluorescence analysis. The antigen levels were calculated upon the dilutions made and showed promising results (Table 6.8) which were backed by a previous study using radioactively labelled H3N2 antigen (41.6 ± 5.27 % - Chapter 5).

Table 6.8: Antigen association data showing antigen is present after the freeze drying cycle had taken place for niosome formulations (5:4:1 MPG:Chol:DCP respectively) (n=3).

Formulation	Antigen entrapment (%)
Control- Nisv	38.87 \pm 2.49
Capsule- Nisv	30.43 \pm 3.37
Fast Melt- Nisv, 10% dextran 10% Mannitol	36.92 \pm 1.80
Oral Chewable- Nisv, 10% dextran 10% Mannitol 1% v/v Strawberry	46.96 \pm 4.51

Table 6.8 presents the results from the antigen incorporation where the data is reproducible as the antigen was recovered after rehydration of the tablets and capsules. This study confirmed that the antigen retained with the vesicles is backed by a previous study using the lower melt method which resulted in an antigen association of 41.6 \pm 5.27 % (Chapter 5). The oral chewable tablet showed higher levels (approx 10 % greater) of antigen association compared to the control and other formulations which was expected due to the oral chewable tablets being smooth and compact in relation to the fast melt tablets. This effect was previously observed in section 6.3 where the addition of the strawberry flavour had adhesive glue like properties. The addition of strawberry flavouring exhibited adhesive properties and thus the increased antigen association could be related to the presence of the strawberry flavour keeping the potential free antigen locked around the vesicles as well as residing within the vesicles. Generally, the increased antigen content is beneficial as improved antigen loading offers a greater chance for more antigen to be present at the Peyer's patches via the oral route as shown in Chapter 5.

6.6 Conclusion

Within this chapter, the exploitation of the freeze drying process by the addition of excipients to niosome vaccine preparation results in the production of alternative solid dosage forms for oral delivery of vaccines. The results demonstrated that prior to

adding mannitol and dextran to the niosomes the average VMD of the vesicles was 6 μm and upon addition of excipients decreased to 4-6 μm which was a result of the excipients bringing the VMD down and not so much the vesicles reducing in size. Upon rehydration after freeze drying the vesicle VMD averaged out to 4-5 μm with the zeta potential remaining unchanged. Tablet hardness studies showed that the increase of dextran to 10 % increased tablet hardness, with toughest tablet resulting from the addition of 1 % strawberry flavouring due to its adhesive nature hence forming a glaze over the tablet and causing the compaction of the product thus resulting in increased hardness. The disintegration time varied upon the hardness of the tablets which showed a trend that the harder tablets (10 % dextran) took longer to disintegrate than the 5 % dextran tablets. Moreover, when carrying out TGA analysis, all tablets and preparations prepared passed the moisture content tests where no more than 5 % residual moisture was present. Comparing the disintegration time, hardness and moisture content; the higher moisture content within the tablets resulted in lower hardness of the tablets and showed faster disintegration time. TGA analysis showed that to further reduce residual moisture a secondary drying phase at 50 °C could be added to the freeze drying cycle potentially removing an extra 1.5 % of residual moisture. However, it is to be noted that sealing of tablet vials occurred outside the freeze dryer (at room temperature and pressure) and residual moisture could have been introduced within the samples from the exposure to atmospheric conditions and humidity.

In conclusion, the current work in producing an alternative to a liquid dosage form of a niosome vaccine delivery system has shown great promise by reformulation into tablets and capsules. The tablets and capsules offer a broader spectrum of delivery and can be classed due to their disintegration times. The dosage forms produced within this study can be classed into four major categories which include:

- Oral dispersible pouch (Control),
- Rapid fast melt (less than 10 seconds),
- Oral chewable tablet (Greater than 30 seconds),
- Prolonged release capsules (Greater than 5 minutes).

The use of 10 % mannitol and 10 % dextran produced hard and durable tablets which offered strong resistance to mechanical damage and protection of vesicles during transit. The antigen association studies using fluorescence proved that the antigen remained with the tablets after the lyophilisation. The freeze drying process was confirmed with the study from chapter 4 where the trivalent fluzone vaccine based niosomes were freeze dried using the same conditions and an immune response was detected in Balb/C mice hence the antigen remained stable after freeze drying. The antigen association studies confirmed that approximately 30-40 % antigen is able to associate itself with the niosome vesicles prepared via the Aston melt method. To conclude, this chapter confirms several dosage designs where niosome delivery systems are a suitable platform to administer a wide range of drugs/antigens (Potential for poorly soluble drugs) via this route where the freeze drying process increases shelf life and stability of the formulations.

Chapter 7

Final Discussion and Future Work

7.0 Final discussion

The fundamentals of this thesis were to exploit non-ionic surfactant technology for delivery and administration of flu vaccine antigens across various routes and to gain a better understanding of vaccine trafficking and immunity. This thesis has demonstrated that the redevelopment of a niosome preparation method after conducting extensive optimisation has overcome current problems associated with present niosome production techniques. The proposed Aston melt method within this thesis overcomes problems whereby previously, antigen integrity would be compromised due to the high process temperatures and the location of antigen in respect to the niosome vesicles offered less protection to the antigen. As a result, the Aston melt method for production of niosome vesicles is able to allow greater levels of antigen to be entrapped within the niosome vesicles based on radioactive and trypsin digestion studies thus offering protection to the antigen from the external milieu of the GIT or plasma. In turn, providing thermostability to the antigens upon storage at elevated temperatures.

The method development of the Aston melt was successful due to the vast optimisation studies carried out which included various techniques that provided an insight into surfactant behaviour, thermodynamics of the vesicles, stability studies, and characterisation data. To start, the implications of varying surfactant ratios upon responses such as suspension pH, vesicle size, vesicle surface charge and peptide entrapment within non-ionic surfactant vesicles was determined. Results showed that bilosome manufacture can be optimised by controlling specific formulation parameters such as homogenisation head type, speed and by keeping assays consistent. DoE was used to plan and conduct experiments in a random order and used to extract the utmost information from the data obtained. The aim of designing

an experiment through MODDE was to gather data in the fewest number of experimental runs, therefore, saving time and resources where the factors in the experiment were simultaneously varied. The aim of DOE was to create a database whereby analysis using a mathematical model was calculated where the data was then interpreted and predictions made which resulted in the optimisation of the surfactant ratios for optimum attributes for uptake at the site of action; the Peyer's patches.

In terms of optimising the surfactant ratios, analysis from the DOE studies reflected that DCP was the key lipid which influenced the surface charge which was relative to the pH of the suspension. The vesicle size was influenced by the addition of bile salts within the formulation which was also dependant on the concentration of bile salt added. Based on criteria for vesicles to be successfully taken up by the Peyer's patches with optimum vesicle size of $> 4\mu\text{m}$ with a highly negative zeta potential (Tabata and Ikada, 1988), pH between 7-9 and highest antigen entrapment it was predicted that a surfactant ratio of 5:4:1 of MPG:Chol:DCP respectively should be used for niosome vesicles with the addition of 70-120 mM bile salt for bilosome vesicles. This optimum ratio of surfactants was then further validated by a series of experiments to confirm optimum vesicle characteristics for uptake based on literature and within this thesis the 5:4:1 ratio of MPG:Chol:DCP respectively was determined to be the optimum formulation for future studies.

Upon determination of surfactant ratios for optimised physical characteristics the thermodynamic stability and surfactant interactions based on Langmuir monolayer, differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) studies were carried out. Thus, studies using DSC, TGA and Langmuir monolayer isotherms were conducted to gain a further insight into the behaviour of the

surfactants at the ratio of 5:4:1 MPG:Chol:DCP respectively. Initial TGA studies showed that the individual components were stable above 200 °C and that when combined in the mixture at the operating temperatures ~2 % lipid degradation was observed however, lowering the melt temperature was able to overcome this issue where no degradation occurred when melting the lipids at 95°C. In terms of monolayer isotherms, the excipients were all in line with literature values and at the specified ratio of 5:4:1 of MPG:Chol:DCP respectively the experimental and calculated values showed no significant differences thus implying that the mixture was homogenous. This was then backed up by DSC studies confirming the presence of cholesterol within the bilayer vesicles due to the abolishment of the gel-liquid crystalline transition temperature.

The optimised niosome formulation by the various characterisation techniques and analytical techniques demonstrated that the niosome vesicles are stable and are able to retain antigen. It has been demonstrated that the advantage of using delivery systems is that the antigens can also be protected after administration (Gregoriadis et al., 1999, Gregoriadis, 1994). The growing concern over the thermostability of vaccines globally, has been one of the key areas of this research to determine whether the niosomes produced offer thermostability in addition to allowing a reduction within the doses used. As previously discussed, the development of the Aston melt niosomes have shown that the niosome vaccine delivery system was able to protect the vaccine with no loss in potency for a period of 3 months at 40 °C, thus overcoming the global problems associated with power outages and temperature increases worldwide.

In terms of oral delivery of vaccines, research has demonstrated that antigen administration via the oral route is insufficient to elicit sufficient immune responses thus require the use of adjuvants and delivery systems to enhance immunity (Azizi et al, 2010). Hence the proposed method of producing vesicles to protect thermolabile antigens by lowering the process temperatures (Aston melt) showed that antigen associated and entrapped within non ionic surfactant vesicles offer increased protection from GI fluids. Digestion studies demonstrated that encapsulated antigen is protected and when the oral biodistribution was conducted, the presence of the delivery system holding the antigen lead to increased recovery of antigen and carrier along the GIT with increased levels at the target sites of the Peyer's patches and the mesentery. Uptake was confirmed *in vivo* by the use of fluorescent markers and Confocal microscopy which expressed that the vesicles remained intact and were spherical and present deep within the Peyer's patches. Studies within this thesis demonstrated that on average 1.5 % antigen is taken up by the Peyer's patches, whether this quantity of antigen is enough to elicit strong immune responses need to be confirmed by vaccine efficacy studies. Furthermore, in addition to the low efficacy associated with administering free antigen alone, the antigen uptake is also dependant on the size of the carrier. This thesis demonstrates that the larger vesicles (~ 6 μm) are able to reside within the Peyer's patches compared to the corresponding smaller vesicles (~ 2 μm) thus is an indicator of possibly providing increased mucosal immunity. However, the studies conducted (Chapter 5) show that it is important to control and understand dosing and antigen concentrations administered as increased dose concentration may not be commercially viable with the increased chance of reduced immunity due to oversaturation and increased systemic tolerance.

Hence, it is vital to carry out a dose dependant vaccine efficacy study within the future to determine limits for antigen dosing.

One of the growing concerns globally is the logistics of vaccine delivery to various parts of the world which would require trained personnel for vaccine immunisations. One of the aims of this thesis was to develop an alternative dosage design from needle based vaccines to developing needle free vaccines. Chapter 6 demonstrated the vast potential of lyophilising niosome delivery systems whereby, successful tablet and capsule based formulations were produced. The tablets produced were classed as rapid fast melts which exhibit patient compliance with the advantage of aiding global immunisation strategies by allowing the removal of cold chain storage and trained personnel. Figure 7.1 demonstrates the current options available for niosome based vaccines based on the work within this thesis.

7.1 Final Conclusions

To summarise, exploitation of non ionic vesicles as carriers of flu vaccine antigens demonstrated that:

- The 5:4:1 ratio of MPG:Chol:DCP respectively offer the optimum characteristics for vesicle size, zeta potential, pH and antigen retention for uptake via the oral route.
- The niosome vesicles prepared, provide thermostability to thermolabile antigens for a period of 3 months exposed to elevated temperatures and humidity whereby upon administration intramuscularly, showed immune responses comparable if not greater than the control commercial vaccine stored in the fridge.

- Niosome vesicles containing bile salts within their construct has shown to induce efficacy in an oral ferret challenge study whereby responses achieved were comparable to the commercial vaccine administered intramuscularly.
- Niosome vesicles are able to be formed as alternative dosage forms other than liquids such as fast melt tablets, oral chewable tablets, and capsules which maintain vesicle integrity and antigen, not requiring the use of cold storage.

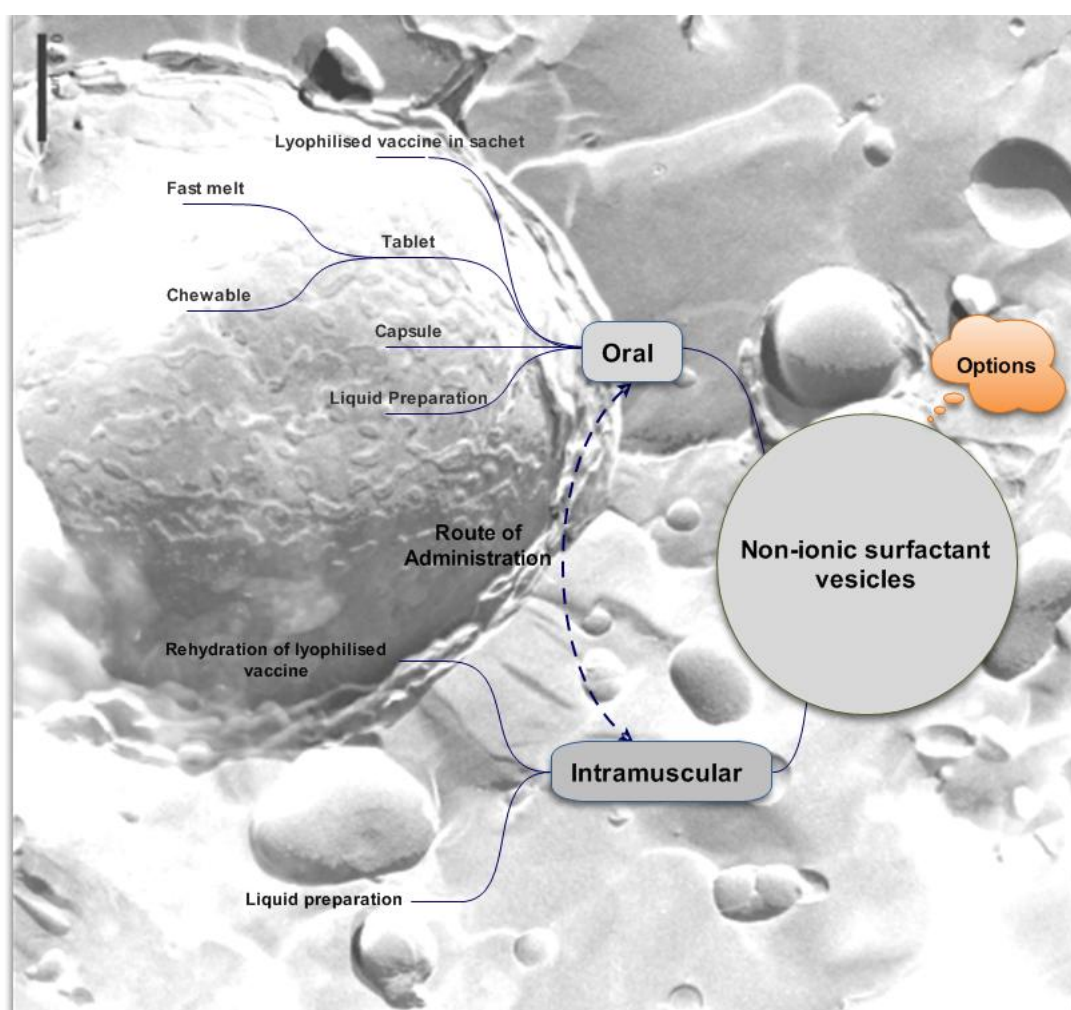


Figure 7.1: Current options for delivery of niosome vesicles via oral or intramuscular delivery demonstrating the possible dosage forms available for the delivery of sub-unit influenza antigens.

7.2 Future work

This thesis has laid the foundations for a great future in the use of non ionic surfactant vesicles as delivery systems of antigens via various administration routes.

One of the key studies for the future would be to determine efficacy via the oral route and to see if mucosal and systemic immunity can be obtained in parallel by optimising the dose administered. Chapter 5 demonstrated that the vesicles prepared are able to reach the target site of the Peyer's patches and that the larger vesicles are able to reside within them thus potentially boosting mucosal immunity. Hence, a vaccine efficacy study would prove whether this can be achieved based on the opinions within literature. A vaccine efficacy implementing several design considerations which include determining responses of niosome versus bilosome vesicles, effect of vesicle size on type of immunity achieved and determination of large and small vesicle size doses in trying to boost mucosal immunity and gain systemic immunity via the smaller vesicles. Techniques such as flow cytometry, *in vitro* M cell uptake with dendritic cell studies should be implemented to provide further insight into cytokine production, the induced immune responses and T cell differentiation.

Moreover, the lyophilisation of the niosome vesicles has shown to significantly provide thermostability to flu vaccine antigens (Chapter 4) and the development alternative dosage forms via tablets or capsules (Chapter 6) has also shown that the delivery systems are able to remain stable and retain antigen. Thus, this provides an avenue to explore the use of a broad spectrum of antigens for various disease states and the possibility of using multiple antigens within the vesicles to reduce numbers of vaccines required. Long term storage (6 months - 1 year) of the thermostable niosome vesicles should be conducted to gain an insight into possible shelf life timescales for the vaccine delivery systems.

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